XENOBIOTIC EFFECTS ON GENE EXPRESSION IN ENDOMETRIAL CELLS AND
PLACENTAL TISSUE

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

This dissertation is dedicated to the memory of my mother

VALERIE LOORIN CHARLES

TABLE OF CONTENTS

Ah	aryl hydrocarbon
AhR	aryl hydrocarbon receptor
Arnt	aryl hydrocarbon nuclear translocator
B(a)P	benzo(a)pyrene
BBE	bovine brain endothelial
βNF	β-napthoflavone
BSA	bovine serum albumin
c-myc	cellular myc ribonucleic acid
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CIAP	calf intestinal alkaline phosphatase
CYP1A1	cytochrome P450 1A1
CYP1B1	cytochrome P450 1B1
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DIM	3,3'-diindolylmethane
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRE/XRE	dioxin responsive element/xenobiotic responsive element
DTT	dithiothreitol
ECM	extracellular matrix

EGF	epidermal growth factor
FBS	fetal bovine serum
GH	growth hormone
GnRH	gonadotropin releasing hormone
hCAII	human carbonic anhydrase II
hr	hours
hsp	heat shock protein
IC3	indole-3-carbinol
IGF-II/M6P	insulin-like growth factorII/mannose-6-phosphate
IUGR	polyaromatic hydrocarbon
kb	kilobases
kDa	kilodaltons
3-MC	3-methylcholanthrene
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTX	methotrexate, aminopterin
MXC	methoxychlor
MW	molecular weight
NLS	N-lauroylsarcosine
PAH	polyaromatic hydrocarbon
pAMBS	p-aminomethylbenzenesulfonamide
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
4-PeCDF	2,3,4,7,8-pentachlorodibenzofuran
PKC	protein kinase C
PL	placental lactogen

PLF	proliferin
PLP	prolactin-like protein
PMSF	phenyl-methyl sulfonyl fluoride
PRL	prolactin
PRP	proliferin-related protein
RNA	ribonucleic acid
SEM	standard error of mean
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TeCB	tetrachlorobiphenyl
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator

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December 1997

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This study evaluated, firstly, the potential role of the environmental contaminants 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo(a)pyrene [B(a)P] in uterine disease utilizing an endometrial adenocarcinoma cell line RL95-2. Secondly, this research study investigated the potential lactogenic and angiogenic activity of two members of the prolactin/growth hormone family, prolactin-like proteins B and C which are secreted by the rat placenta during pregnancy.

TCDD and B(a)P were evaluated for their ability to alter the expression of growth factor and cytokine genes including epidermal growth factor (EGF) receptor, urokinase plasminogen activator (uPA), interleukin (IL-1β) and tumor necrosis factor (TNF-α). This study demonstrated that both TCDD and B(a)P induced the expression of CYP1A1 in RL95-2 cells, but only B(a)P significantly decreased EGF receptor expression. TCDD but not B(a)P significantly increased the steady state level of uPA messenger ribonucleic acid (mRNA), however neither chemical was able to significantly alter the associated fibrinolytic activity of conditioned medium from treated RL95-2 cultures. Furthermore, TCDD

increased the mRNA expression level of TNF-α in a time-dependent and IL-1 β in a time- and dose-dependent manner. Finally, B(a)P, but not TCDD, was able to inhibit significantly, the overall proliferation and invasiveness of these endometrial adenocarcinoma cultures. These results indicate that TCDD and B(a)P can alter the gene expression of members of the growth factor/cytokine network in uterine tissue and so potentially contribute to the promotion of uterine disease.

Native preparations of PLP-B and PLP-C purified from the conditioned medium of placental explant cultures exhibited little lactogenic activity relative to ovine prolactin, as evaluated by their ability to stimulate the proliferation of the rat Nb2 lymphoma cell line. Conditioned media from gestation day 18 rat placenta significantly increased the directional migration of human retinal endothelial cells, a measure of angiogenic activity. In contrast, neither immunopurified native PLP-B nor PLP-C proteins showed any significant stimulation of endothelial cell migration. Both PLP-B and PLP-C were successfully expressed as recombinant proteins in mammalian and bacterial systems. Recombinant PLP-C, in contrast to the native preparation, did not exhibit any measurable lactogenic activity. In summary, the angiogenic activity exhibited by conditioned medium of rat placental cultures is not associated with either PLP-B or PLP-C.

CHAPTER 1
INTRODUCTION

Study Objectives

This research was undertaken to investigate the potential role of environmental contaminants in uterine disease and altered placental function. The objectives of the projects were to investigate the potential mechanisms whereby prototype xenobiotics might (a) potentially contribute to the promotion of uterine disease in general, and endometriosis in particular, as well as (b) alter placental/fetal growth and development. Firstly, recent evidence indicates that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other environmental pollutants such as methoxychlor (MXC) may contribute to the promotion of uterine endometriosis, a benign proliferative disorder (Cummings and Metcalfe, 1995; Gerhard and Runnebaum, 1992; Koninckx et al., 1994; Mayani et al., 1997; Rier et al., 1993). It is now recognized that TCDD and other xenobiotics, which are capable of binding to a cytosolic receptor designated the arylhydrocarbon receptor (AhR), can also act as endocrine and growth modulators via alterations in the expression of a number of genes. Endometriosis is an enigmatic disorder, however, with little being known regarding its histogenesis and maintainence. Our hypothesis for this investigation is that TCDD and benzo(a)pyrene B(a)P are able to alter the expression of uterine genes and gene products involved in implantation and immune responsiveness which can contribute to uterine disease etiology.

Secondly, our laboratory has previously shown that gestational exposure of pregnant rats to β-napthoflavone (βNF) and 3-methylcholanthrene (3-MC), prototypical

polyaromatic hydrocarbons (PAH) and AhR ligands, resulted in intrauterine fetal growth retardation (IUGR) and decreased placental function (Fuhrman-Lane et al., 1983; Shiverick et al., 1984). Furthermore, βNF treatment was found to be associated with the decreased secretion of a family of placental prolactin-like proteins (PLPs) into the conditioned media of placental explant cultures (Shiverick et al., 1991). These rat placental prolactin-like proteins have become of particular interest based upon the recent demonstration of the angiogenic and angiolytic activity of two potentially homologous proteins in murine placenta (Jackson et al., 1994). Thus, these data led us to hypothesize that βNF-mediated alteration in the expression of rat placental PLPs could result in a decreased ability of the placenta to develop a vascular network capable of maintaining adequate fetal growth. To investigate this possibility, we purified native rat placental PLP-B and PLP-C as well as expressed recombinant forms of these proteins in bacterial and mammalian systems in order to evaluate them for potential lactogenic and angiogenic activity. The objective of this part of my research has been to elucidate the potential role(s) of these placental members of the PRL-GH family during the course of pregnancy.

Uterine Disease: Association with Xenobiotic Agents

There has been increasing public and scientific concern that environmental pollutants may be able to disrupt the normal hormonal milieu in humans and animals leading to disease pathologies. Many naturally occurring and man-made chemicals present in the environment possess estrogenic and antiestrogenic activity. These include plant and fungal products, pesticides, plasticizers, and other industrial and agricultural chemicals (Stancel et al., 1995). Reports of abnormal sexual development in reptiles (Guillette et al., 1994) and birds (Fry, 1995) have provided evidence to support the proposal that select environmental chemicals function as endocrine modulators. Concerns continue to grow regarding the potential involvement of these agents in such reproductive abnormalities as

breast cancer, endometriosis, fibroids and uterine adenocarcinoma in women (Newbold, 1995).

The growth response of the uterus to steroid hormones is a highly regulated process in reproductively cycling women. Hence this is a potential site for the manifestation of the endocrine disruptive effects of environmental pollutants. Studies in rodents exposed to diethylstilbestrol (DES), a potent synthetic estrogen, during the prenatal and neonatal period showed epithelial and stromal stimulation of the uterine horns, cystic endometrial hyperplasia, as well as a low incidence of benign (leiomyoma) and malignant (adenocarcinoma) uterine tumors (Newbold et al., 1990; Newbold, 1995). This evidence supported the conclusion that developmental exposure to estrogen affects the pattern of uterine cell differentiation, resulting in later morphological and neoplastic alterations.

A number of environmental pollutants have been shown to modulate the growth and differentiation of uterine tissue in both *in vitro* and *in vivo* models. The pesticides dichlorodiphenyltrichloroethane (DDT) and MXC stimulated DNA synthesis in primary cultures of uterine epithelial and stromal cells respectively (Tiemann et al., 1996). The administration of o,p'-DDT to immature rats mimicked the effects of estrogen by increasing uterine weight, as well as by stimulating DNA synthesis and cell division which led to hyperplasia in the uterine luminal epithelium, stroma and myometrium (Kupfer, 1981; Robison et al., 1985). Similarly, the polychlorinatedbiphenyl (PCB) Arochlor 1221 was found to induce uterine growth in neonatally exposed rats (Gellert, 1978).

Some environmental agents, however, exhibit activity which opposes the effects of estrogen. The characteristic example is TCDD which has been demonstrated to have antiestrogenic properties. TCDD exposure has been linked with a variety of antiestrogenic responses in the female rat uterus including inhibition of constitutive and 17β-estradiol-induced uterine wet weight increase, diminished nuclear and cytoplasmic estrogen and progesterone receptor levels, as well as decreased epidermal growth factor (EGF) receptor binding, EGF receptor and EGF receptor mRNA levels, and c-*fos* proto-oncogene mRNA

levels (Astroff et al., 1990, 1991; Romkes et al., 1987, Romkes and Safe, 1988). TCDD has also been associated with a decreased age-related incidence of tumors of the uterus (Kochiba et al., 1978). It should be noted, however, that low dose, short-term admistration of TCDD did not appear to alter the uterotrophic response to exogenous estrogen in ovariectomized rats (Shiverick and Muther, 1982). The PCB congener 3,3',4,4'-TeCB was reported to antagonize the uterotrophic effects of estradiol in the immature female Sprague-Dawley rat (Jansen et al., 1993), while neonatal exposure of female Wistar rats to B(a)P was found to significantly reduce uterine estrogen receptor density in adulthood (Csaba and Inczefi-Gonda, 1993).

It has been hypothesized that these environmental pollutants could potentially contribute to an increased incidence of uterine disease in the female population based upon their ability to modulate the growth and differentiation of uterine tissue. Evidence from epidemiological and laboratory studies is beginning to implicate a number of these environmental toxicants in the etiology of uterine disease pathologies. For example, epidemiological data indicates that cigarette smoking is linked with a modest decrease in risk for endometrial hyperplasia, while smoking women have a risk for endometrial cancer less than half that of non-smokers, an effect more pronounced in postmenopausal compared with premenopausal women (Baron et al., 1990; Baron, 1996). Epidemiological data also suggest that there is an inverse association of cigarette smoking with uterine fibroids and endometriosis (Baron, 1996; Cramer et al., 1986; Matorras et al., 1995). There is presently insufficient data, however, to support a conclusion regarding the effects of organochlorine compounds on endometrial cancer (Ahlborg et al., 1995).

Recent studies in rhesus monkeys and rodents indicate that TCDD and MXC may act as promoters in the development of endometriosis (Cummings et al., 1996; Cummings and Metcalfe, 1995; Rier et al., 1993). In rhesus monkeys, dietary intake of TCDD was associated with an increased incidence and severity of endometriotic lesions (Rier et al., 1993). In surgically-induced rodent models of endometriosis, both TCDD and MXC

supported the development and growth of the endometriotic implants (Cummings et al., 1996; Cummings and Metcalfe, 1995). In addition, human epidemiological evidence is accumulating to support an association between TCDD and the promotion of uterine disease (Koninckx et al., 1994; Mayani et al., 1997). Women with endometriosis were also reported to have increased concentrations of PCBs in their blood (Gerhard and Runnebaum, 1992). Experiments in rhesus monkeys with the PCB Arochlor 1254, however, concluded that the incidence and severity of endometriotic lesions observed in the animals did not have any relation to the doses of PCB ingested during the study (Arnold et al., 1996). Thus, the data are not conclusive and may reflect the different structure of organochlorine compounds as well as their routes of metabolism.

Endometriosis: Disease Histogenesis, Etiology and Promotion

Endometriosis, the term first coined by Sampson (1921), is defined as the presence of glands and stromal tissue, histologically similar to endometrium, outside the uterine cavity and myometrium which is associated with vascularization and cellular proliferation. The most common location of endometriosis is in the pelvis with greatest frequency occurring in the ovary (Jenkins et al., 1986). The symptoms usually observed include the presence of pelvic masses, pain, and infertility (Barbieri, 1992; Olive and Schwartz, 1993). In fact, a recent study indicates that 80% of laparoscopies for infertility result in diagnosis of this disease (Thomas and Prentiss, 1992), leading to 400,000 hysterectomies being performed in the U.S.A in 1984 (Natl. Cen. Health Stat. 1986). Although the exact incidence has been difficult to determine due to the asyptomatic nature of the disease which requires laparoscopy or surgical visualization for a definitive diagnosis, a prevalence of 10% has been estimated in the general female population (Olive and Schwartz, 1993).

Evidence from epidemiological and clinical studies, as well as work on animal disease models, suggests that environmental agents as well as other factors may have a significant role to play in the etiology and pathogenesis of the disease (Cramer et al., 1986;

Gerhard and Runnenbaum, 1992; Koninckx et al., 1994; Matorras et al., 1995; Mayani et al., 1997; Rier et al., 1993). The main etiological factors appear to be exposure to estrogen and the process of cyclic shedding of the uterine lining. In this regard, endometriosis is more common in women with short menstrual cycle lengths (< 27 days) and longer flow periods (> 1 week) (Cramer et al., 1986; Matorras et al., 1995), while occurring infrequently before puberty or after menopause. These studies further reported that endometriosis was less common in smokers and in women with a low body mass index, both factors which lead to lower endogenous estrogen stimulation. Recently, exposure to environmental contaminants like TCDD and PCBs have also been associated with an increased disease incidence in animal and human studies (Gerhard and Runnenbaum, 1992; Mayani et al., 1997; Rier et al., 1993).

The physiological and molecular mechanisms whereby endometriotic tissue develops and persists outside the uterine cavity are poorly understood and remain controversial. Endometriosis has been described as the disease of theories, there being several existing hypotheses as to its pathogenesis. Three main concepts predominate (as reviewed in van der Linden, 1996; Olive and Schwartz, 1993). The *in situ* development theory states that endometriosis develops in the location where it is found, thought to be the consequence of metaplasia of peritoneal or ovarian tissue (Lauchlan, 1972). This theory does not, however, explain why the disease occurs exclusively in women, in the pelvic organs, during their reproductive years. The second concept, the induction theory, argues that endometriosis results from the differentiation of mesenchyme induced by substances released by degenerating endometrium (Merrill, 1966). The third theory of histogenesis is that uterine endometrium is transplanted to ectopic locations through lymphatic and vascular dissemination and retrograde menstruation (Sampson, 1927). The implantation theory is at present the most widely held with the anatomical patterns of the disease being consistent with retrograde menstruation (Jenkins et al., 1986). The implantation theory, however, does not account for the fact that retrograde menstruation occurs in the vast majority of the

female population, whereas endometriosis develops in only a fraction of the these women. This inconsistency has led to the postulate that an impaired immune response or response to tissue injury may result in the inability to remove refluxed menstrual debris, thereby increasing the possibility of endometriosis (Dmowski et al., 1994; Gleicher and Pratt, 1993).

Mechanisms of Action of TCDD and B(a)P

Ah Receptor Activity

The aryl hydrocarbon receptor (AhR) mediates most of the toxicological effects of certain halogenated aromatic hydrocarbons that are widely disseminated in the environment, including TCDD and polyaromatic hydrocarbons (PAHs) found in cigarette smoke and industrial exhaust (Hankinson, 1994; Whitlock, 1993). The unliganded AhR is a basic-helix-loop-helix protein which resides in the cytoplasm in association with the 90 kDa heat shock protein (hsp90) and functions as a ligand-activated transcription factor (Hankinson, 1994; Whitlock et al., 1996). While TCDD and related PAHs like B(a)P are known to bind the receptor, little is known regarding the binding of physiological ligands to the Ah receptor. Compounds like indole-3-carbinol (IC3) and 3,3'-diindolylmethane (DIM) found in cruciferous vegetables, however, have been shown to activate the AhR, although they possess a much lower receptor affinity (Jellinck et al., 1993; Kleman et al., 1994). The AhR has been implicated as the primary mediator of toxicity of the PAHs based on evidence that the toxicity of individual congeners is correlated with affinity for the receptor, as well as that susceptibility to a range of toxic effects segregates with the *Ah* allele in highly responsive mouse strains like C57BL/6 (Okey et al., 1994).

The binding of ligands like TCDD is thought to result in allosteric changes in the dimerization domain of the AhR which allows for release of the hsp90, followed by translocation to the nucleus and interaction with a nuclear protein, the arylhydrocarbon nuclear translocator (Arnt). There is some evidence which implicates protein kinase C

(PKC) and tyrosine kinase in the generation of an active AhR/Arnt complex, yet the precise role of these kinases remains unclear (Gradin et al., 1994; Schafer et al., 1993). The heterodimeric AhR:Arnt complex, possibly in association with other proteins/factors (Chan et al., 1994; Dunn II et al., 1996), is able to bind to enhancer sequences termed dioxin or xenobiotic response elements (DREs or XREs) upstream of structural genes such as CYP1A1. Xenobiotic-induced binding of the AhR/Arnt complex to enhancer chromatin sequences is then associated with localized changes in chromatin structure manifested by increased accessibility of the gene promoter DNA sequence to general transcription factors such as the TATA-binding protein. The initiation complex formed at the promoter then allows for gene transcription to be initiated (Okino and Whitlock, 1995; Whitlock et al., 1996).

The principal route of AhR mediated toxicity is thought to occur via the ability of the AhR/Arnt complex to facilitate the increased transcription of metabolic enzymes like CYP1A1 leading to the bioactivation of pretoxicants to their reactive metabolites, which may result in cytotoxic, carcinogenic or teratogenic effects. In addition, these regulated enzymes may also play a role in the metabolism of endogenous compounds involved in the control of cellular growth and differentiation (Hankinson, 1994; Okey et al., 1994). The fact that TCDD, B(a)P and related compounds have been demonstrated to alter gene expression in uterine tissue, coupled with the ubiquity of AhR tissue expression (Dolwick et al., 1993), makes it feasible to investigate the potential action of these compounds in the promotion of uterine pathologies.

AhR Independent Mechanisms

Some scientists question that AhR-mediated induction of gene transcription is the sole route for the toxic effects of TCDD and related compounds. Recent work under cell-free conditions has demonstrated that TCDD is able to activate protein kinases in the absence of a nucleus (Enan and Matsumura, 1995). Induction of immediate-early response

protooncogenes like *c-fos* and *jun*B by TCDD and B(a)P appears to be independent of AhR and Arnt in variant hepatoma cell lines (Hoffer et al., 1996; Puga et al., 1992). This evidence lends support to the possibility of distinct signal transduction pathways for the mediation of the toxic effects of these compounds. Furthermore, the toxic effects of B(a)P may be exacerbated by metabolism in mammalian cells to reactive products that can covalently bind to DNA, as well as through the generation of reactive oxygen species capable of producing direct cellular damage (Leadon et al., 1988). TCDD is itself highly resistant to metabolism and has an approximate 7-10 year half life in humans. These differences in mechanisms of action may account for the observed differences in the epidemiological findings of cigarette smoking compared with TCDD as relates to uterine disease (Ahlborg et al., 1995). Our study of their comparative effects in our endometrial culture system should further the understanding of these empirical observations.

Use of Endometrial Carcinoma Cells for the Study of the Potential Role of Xenobiotics in Endometriosis

The question sometimes arises as to the appropriateness of utilizing *in vitro* cultures of transformed cells as models for the study of disease pathologies. In this regard, a major concern is the potential difference in responsiveness of transformed cells compared to the original uterine tissue. The use of primary endometrial cell culture, however, presents a number of problems, such as the cyclical hormonal variation which exists throughout the menstrual cycle, the lack of tissue homogeneity, finite life span, interindividual variation of the tissue, and potential loss of steroid and other receptor signalling systems (Tabibzadeh et al., 1990; Watson et al., 1994). The use of tissue from patients with endometriosis presents similar difficulties with patient variability in severity of this disease, as well as that of the availability of a reliable supply of tissue for culture maintainence.

The endometrial adrenocarcinoma RL95-2 cell line was established by Way et al (1983) and has been investigated with respect to the action of several growth factors including EGF, TGFα and TGFβ (Korc et al., 1986, 1987). Liu and Teng (1994) demonstrated that estrogen was able to produce measurable responses in the RL95-2 cell line. Similarly, Grenman et al (1988) demonstrated RL95-2 responsiveness to both estrogen and progesterone. RL95-2 also exhibits significant EGF and TGFβ binding activity (Dumont et al., 1995; Korc et al., 1986; Lelle et al., 1993). Endometriotic tissue from patients transcends the clinico-pathologic distinction between a benign disease and an invasive neoplasm. While histologicaly benign, endometriotic tissue invades local pelvic structures (Koninckx and Martin, 1992). Hence we considered the use of the adenocarcinoma RL95-2 cell line to be appropriate as an in vitro model for the purposes of the present study.

PRL/GH Related Rrotein Expression in Rodent Placental Tissue

Placental Prolactin Family of Proteins

The rodent develops two distinct placental structures during the course of gestation. The first to develop is the choriovitelline placenta which disappears by day 14 of gestation. While the choriovitelline placenta is degenerating, the chorioallantoic placenta comes into existence being composed of three major regions, the labyrinth (60%), the basal or junctional zone (15%), and the decidua basalis, subplacental region and metrial gland (25%) which comprise the remaining part of the total placental structure late in gestation (Davies and Glasser, 1968). The rodent placenta is a rich source of placental lactogens (PLs) and several other proteins which have a structural homology to pituitary PRL rather than to GH (Soares et al., 1991; Southard and Talamantes, 1991). It is predominantly in the basal or junctional zone that the PRL-like proteins are expressed.

Amino acid or nucleotide sequence data is now available for fifteen of these proteins. The seven placental lactogens include rat and mouse PL-I (Colosi et al., 1987a; Robertson et al., 1990), rat PL-Iv (Deb et al., 1991; Robertson et al., 1991), rat PL-I mosiac (Hirosawa et al., 1994), hamster, rat and mouse PL-II (Duckworth et al., 1986a; Jackson et al., 1986; Southard et al., 1986). The seven prolactin-like proteins include: mouse Proliferin (PLF) and Proliferin-Related Protein (PRP) (Linzer and Nathans, 1985; Linzer et al., 1984), and rat prolactin-like proteins A, B, C, Cv and D (Dai et al., 1996; Deb et al., 1991c; Duckworth et al., 1986b; Duckworth et al., 1988; Iwatsuki et al., 1996). Rat decidual prolactin-related protein (dPRP), which shows a high degree of sequence identity to PLP-C, has also been cloned from rat decidua (Roby et al., 1993), while a PRL-like cDNA from the midgestation hamster placenta similar to PL-I has recently been isolated and characterized (Barnes and Renegar, 1996). These proteins exhibit both cell and temporal specific patterns of expression, being secreted predominantly by the spongiotrophoblast and trophoblast giant cells of the junctional or basal zone.

Comparison of the amino acid sequences of these proteins indicates that amongst the PL-Is there is over 70% sequence identity and similar homology exists among the PL-IIs. In contrast, the prolactin-like proteins A, B, C, Cv, D, Proliferin (PLF) and Proliferin Related Protein (PRP) have between 12-40% sequence identity with each other and to the PLs (Iwatsuki et al., 1996; Southard and Talamantes, 1991). The structural assignment of these proteins to the PRL family has been based on the positioning of conserved cysteine residues, as well as additional amino acid sequence homologies. Any similarities in biological activities to PRL were not considered a requirement for inclusion, since in most cases these activities were yet to be determined. Therefore, as a group they bear at maximum a 45% sequence homology to pituitary PRL.

Prolactin is known to exhibit a broad range of distinct physiological actions important in reproduction. These include regulation of amniotic fluid volume and ion content, development of the mammary gland and milk protein production, along with

suppressing the immune response and uterine contractility (Handwerger et al., 1992). At the onset of pregnancy, pituitary PRL secretion exhibits a twice daily surge which abruptly terminates at midgestation when the chorioallantoic placenta develops and secretion remains depressed for the rest of gestation (Smith and Neill, 1976). The presence of pituitary PRL after day 6 of gestation is not required for the maintenance of the corpus luteum (Morishige and Rothchild, 1974). It has also been observed that removal of the anterior pituitary in the rat after midgestation did not interrupt pregnancy (Pencharz and Long, 1931). Pituitary PRL could not be replaced by decidual PRL in rodents, as it may be in humans, since rodent decidua does not express this hormone (Handwerger et al., 1984). The secretion by the placenta of high levels of placental lactogens and prolactin-related proteins during the course of pregnancy has led to the hypothesis that they are of importance in the placental-fetal growth axis.

Biological Activities of Placental Proteins

There has been a great deal of interest in determining whether or not these newly discovered proteins were "PRL-like" in their ability to bind the PRL receptor, or in the expression of similar bioactivities. Studies characterizing these rodent placental proteins have demonstrated that some of them do in fact exhibit PRL-like activities. This has been shown by the ability of haPL-II, rat and mouse PL-I and PL-II and rat PL-Iv to exhibit lactogenic activity by stimulating prolactin-like responses in rat Nb2 lymphoma cells (Cohick et al., 1995; Colosi et al., 1987a, b; Deb et al., 1991c; Robertson et al., 1982, 1994), in mammary gland epithelial cell differentiation (Soares et al., 1983; Southard et al., 1986), and in the pigeon crop sac assay (Colosi et al., 1982). Most of the other prolactin-like members of the family have all been expressed, but evidence does not show lactogenic activity (Cohick et al., 1997; Conliffe et al., 1994; Rasmussen et al., 1996). Mouse PL-I and PL-II have also recently been shown to be luteotropic and to support progesterone production in the mouse at midgestation (Galosy and Talamantes, 1995). The daily surges

of pituitary PRL have been demonstrated to be indirectly inhibited by placental lactogen (Tomogane et al., 1992). The hypothesis thus arose that this family of secreted placental members of the PRL-GH family could somehow, perhaps via a feedback mechanism, replace PRL functionally during gestation.

Two members of the placental PRL-GH family, proliferin (PLF) and proliferin related protein (PRP), have recently been shown to display angiogenic and angiolytic activity, respectively (Jackson et al., 1994). PLF and PRP have also been shown to compete with 16K PRL for binding to membranes of bovine brain endothelial (BBE) cells (Clapp and Weiner, 1992). The 14K and 16K forms of PRL, enzymatically generated N-terminal fragments of 23K pituitary PRL, are themselves potent angiolytic factors in BBE cells (Clapp et al., 1993, 1994; Ferrara et al., 1991). Furthermore, PLF has been demonstrated to bind to capillary endothelial cells in the placenta (Jackson et al., 1994), as well as to sites in the developing embryonic vertebral and vascular structures (Jackson and Linzer, 1997). Competition and comparative binding studies indicate that the insulin-like growth factorII/mannose-6-phosphate receptor is involved in PLF binding (Lee and Nathans, 1988; Volpert et al., 1996), as well as to a receptor in uterine membrane preparations of pregnant mice (Nelson et al., 1995). The chemotaxis initiated by PLF and mediated by the IGF-II/mannose-6-phosphate (IGF-II/M6P) receptor appears to occur through a G protein-coupled pathway via MAPK activation. This was demonstrated by the fact that PLF stimulated MAPK activity and endothelial cell chemotaxis, both activities being blocked by pertussis toxin and the specific inhibitor of MAPK kinase, PD 098059 (Groskopf et al., 1997). Although PLP-A, PLP-B, PLP-C and dPRP have been expressed as recombinant proteins, their biological role(s) remain to be elucidated (Cohick et al., 1997; Conliffe et al., 1994; Deb et al., 1993; Rasmussen et al., 1996).

Our laboratory has previously characterized the expression of a number of these placental prolactin-like proteins in the rat placenta (Ogilvie et al., 1990a, b). Observations from our laboratory have associated maternal xenobiotic exposure and protein malnutrition

with the decreased secretion of placental prolactin-like proteins from basal zone explant cultures (Conliffe et al., 1995; Shiverick et al., 1991). A further correlation was noted with decreased placental vascularization and intrauterine growth retardation which has led us to further investigate the potential for two of these proteins, PLP-B and PLP-C, to exhibit lactogenic and angiogenic activity. Although they may not be the homologs of PLF and PRP, they may function similarly, although they appear to lack the conserved amino acid residues essential for lactogenic activity (de Vos et al., 1992; Goffin et al., 1993, 1994, 1996; Somers et al., 1994; Southard and Talamantes, 1991). Angiogenesis is an important factor in placental development. Placental vascular growth begins early in pregnancy and continues throughout gestation, with dramatic increases in fetal-maternal blood flow. Similarly, placental anti-angiogenic factors likely target the maternal placental vasculature and may function to limit vascular development and possible invasion by fetal tissue. Therefore the potential for PLP-B and PLP-C to act as angiogenic or angiolytic factors warrants investigation.

Angiogenesis, Xenobiotics and Placental-Fetal Development

Angiogenesis was first coined as a term to describe the formation of new blood vessels in the placenta (Hertig, 1935). It is the biological mechanism of new capillary formation involving the activation, migration and proliferation of endothelial cells from pre-existing venules (Höckel et al., 1993). Defects in angiogenesis may contribute to a variety of disorders such as endometrial hyperplasia, dysfunctional uterine bleeding, endometriosis, pregnancy loss, pre-eclampsia and cancer (Gordon et al., 1995). The process of angiogenesis has a critical role to play in placental/fetal establishment and development (Reynolds et al., 1992; Welsh and Enders, 1991). Throughout gestation, placental transport capacity keeps pace with fetal growth and uterine blood flow increases approximately three to four-fold from mid to late gestation (Reynolds et al., 1986). Consequently, factors which contribute to inadequate placental vascular development may

have a tremendous impact on fetal growth and development, and, ultimately, on neonatal growth and survival. Embryonic wastage and reduced birth weights are recognized to be major socio-economic problems associated with pregnancy (Reynolds and Redmer, 1995).

The association of environmental agents like cigarette smoke, TCDD and PCBs with the incidence of teratogenicity, low birth weight and fetotoxicity (Couture et al., 1990; McNulty, 1985; Sachs, 1989) may be related to their ability to interfere with the neovascularization process during the course of gestation. Evidence to support this hypothesis has begun to accumulate. For example, pregnant mice exposed to TCDD and 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF) exhibit rupture of the embryo-maternal vascular barrier and the visceral yolk sac membrane, resulting in the hemorrhaging of embryonic blood into the maternal circulation and the uterine and amniotic cavities (Khera, 1992). Similarly, TCDD exposure in bird and fish models leads to pericardial and yolk sac edema and hemorrhaging (Henry et al., 1997; Spitsbergen et al., 1991).

The exact mechanism underlying this pathology is not well understood, but appears to be, at least in part, due to the ability of these agents to cause vascular derangements, possibly via disruption of endothelial cell barrier function, or alternatively, as a result of CYP1A1 induction with resulting oxidative damage (Guiney et al., 1997; Stegeman et al., 1995; Toborek et al., 1995). The possibility that these environmental pollutants may also act through endocrine disruptive mechanisms is highlighted by the fact that functional estrogen receptors are required for the augmentation of basic Fibroblast Growth Factor (bFGF)-induced uterine angiogenesis in female mice (Johns et al., 1996). The effect appears to be an AhR-mediated process as seen by the fact that those compounds which do not bind the AhR, do not produce endothelial cell dysfunction (Toborek et al., 1995). In addition, *Arnt* $^-$ embryos die *in utero* as a consequence of abnormal yolk sac angiogenesis (Maltepe et al., 1997). Finally, CYP1A induction in endothelium in early development in a trout model appears to correlate with mortality and the development of edemas prior to death, a process which is consistent with involvement of the AhR (Guiney et al., 1997).

CHAPTER 2
MATERIALS AND METHODS

Materials

Chemicals and Bioreagents

TCDD was obtained from Midwest Research Institute (Kansas City, MO) through the National Cancer Institute Chemical Carcinogen Reference Repository. Benzo(a)pyrene, aminopterin, insulin, transferrin, endothelial cell growth supplement and ampicillin were purchased from the Sigma Chemical Co. (St. Louis, MO). Ovine prolactin was obtained from the National Hormone and Pituitary Program (NHPP). $[^{125}I]$-EGF and $[^3H]$-methyl-thymidine were purchased from Amersham Life Sciences (Arlington Heights, IL) and $[\alpha-^{32}P]dCTP$ and $[\alpha-^{32}P]UTP$ from ICN Biomedicals Inc. (Irvine, CA). The Prime It$^®$ II random primer labelling kit and Nuctrap$^®$ probe purification columns were purchased from Strategene (La Jolla, CA) and ExpressHyb hybridization buffer from Clontech Laboratories Inc. (Palo Alto, CA). The CellTiter 96™ nonisotopic cell proliferation assay kit was obtained from Promega (Madison, WI). Restriction and modifying enzymes were purchased from Promega (Madison, WI), New England Biolabs (Beverly, CA) or Gibco/BRL (Grand Island, NY). The Fisher Leukostat™ stain was from Fisher Scientific (Lexington, MA). p-Aminomethylbenzenesulfonamide agarose resin (pAMBS) was purchased from Sigma Chemical Co. (St. Louis, MO) and ProBond™ Resin from Invitrogen (San Diego, CA). Cell culture media, Lipofectin$^®$ and antibiotics were from Life Technologies (Gaithersburg, MD) and Sigma Chemical Co (St. Louis, MO), with the exception of fetal bovine serum which was obtained from Hyclone Laboratories (Logan UT). Plasminogen, thrombin and fibrinogen were purchased from CALBIOCHEM$^®$

Biochemicals (La Jolla, CA). Low gelling temperature Sea Plaque Agarose™ was obtained from FMC© BioProducts (Rockland, ME), enterokinase from Biozyme Laboratories International Ltd. (San Diego, CA), G418 sulphate from CELLGRO (Herndon, VA), and bovine dermal collagen and Matrigel from Collaborative Biomedical Products (Bedford, MA). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

Recombinant cDNA Clones, Vectors and Plasmids

Plasmids containing cDNA for EGF receptor (pE7), TGF-α (phTGF1-10-3350), c-myc (pG1-5'-c-myc), CYP1A1 (phP1-450-3'), uPA (pHUK-8), TIMP-1 (pALP-181-EPA57), TIMP-2 (pSS38), TNF-α (pAW739) and β-actin (HHCI89; 65128) were obtained from the American Tissue Type Culture Collection (ATCC) (Rockville, MD). The plasmids containing cDNA for CYP1B1 (Sutter et al., 1994) and IL-1β (Sutter et al., 1991) were kindly provided by Dr. William Greenlee (University of Massachusetts, Worchester, MA). Full length cDNAs for PLP-B and PLP-C (Clone C-308) were generous gifts of Dr. Mary Duckworth (University of Manitoba, Canada) and Dr. Michael Soares (University of Kansas, Kansas City, KS), respectively. For Northern blot analyses, the probes used were a 1.0 kb *Eco*RI fragment for CYP1A1, a 2.4 kb *Cla*I fragment for EGF receptor, a 1.6 kb *Sac*I fragment for c-myc, a 1.5 kb *Pst*I fragment for uPA, a 410 bp *Ava*I/*Hinc*II fragment for TIMP-1, a 790 bp *Eco*RI/*Xba*I fragment for TIMP-2, a 1.5 kb *Eag*I fragment for CYP1B1, a 1.4 kb *Eag*I fragment for IL-1β, a 1.3 kb *Hind*III fragment for TNFα and a 1.1kb *Eco*RI fragment for β-Actin. The mammalian expression vector pMXSND (Lee and Nathans 1988) was generously provided by Dr. Daniel Linzer (Northwestern University, Evanston, IL). The bacterial expression vector pET22b(+) (Novagen, San Diego, CA) and all other non-expressing bacterial strains were provided by Dr. William Farmerie and G. Van Heeke (University of Florida, Interdisciplinary Center for

Biotechnology Research [ICBR], Protein Expression Core, Gainesville, FL). Oligonucleotide primers were synthesized by the ICBR DNA Synthesis Core.

Antibodies and Antisera Generation

A polyclonal sheep anti-human EGF receptor antiserum was acquired from Upstate Biotechnology Inc. (Lake Placid, NY) and the polyclonal goat anti-rat CYP1A1 antiserum from Gentest (Woburn, MA). The horseradish peroxidase labeled goat anti-sheep IgG and rabbit anti-goat IgG were purchased from Bio-Rad Laboratories (Hercules, CA). Rabbit polyclonal antisera against PLP-B was previously generated in our laboratory (Ogilvie et al., 1990a). To generate antiserum against recombinant PLP-C, a New Zealand White rabbit was injected subcutaneously with 220 μg of purified recombinant PLP-C in Freund's complete adjuvant, and three booster injections of 300 μg hCAII-PLP-C fusion in Freund's incomplete adjuvant were administered at 1 week intervals. Two weeks after the final booster, the animal was bled and hCAII cross-reactivity was absorbed out during an overnight incubation with hCAII-pAMBS resin. The hCAII-pAMBS resin was generated by incubating hCAII with pAMBS overnight.

Methods

Cell Cultures and Chemical Treatments

RL95-2 Endometrial Carcinoma Cells   The human endometrial carcinoma cell line RL95-2 was obtained from ATCC and maintained in DMEM:HAM'S F-12 (1:1) supplemented with 10% (w/v) FBS in a humidified atmosphere containing 5% CO_2 at 37°C. All media contained penicillin and streptomycin at 100 μg/ml. Media was changed every 2-3 days, and all experiments initiated when cells were at approximately 50-75% confluence. Cells were cultured in the presence or absence of chemical treatments, added in either DMSO,

ethanol or buffered aqueous solution. Stock solutions of TCDD and B(a)P were prepared in DMSO and added to cultures with final DMSO concentrations at 0.1% (w/v). Appropriate vehicles were added to cultures as controls.

Nb2 cells The Nb2-11c subline was kindly provided by Dr. Paul Kelly (INSERM, Paris, France). It was maintained in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCOBRL, Grand Island, NY), 50 µM 2-mercaptoethanol, 10% (w/v) horse serum (HS) and 10% (w/v) FBS. Starvation media was composed of the maintainence media minus FBS.

Chinese Hamster Ovary (CHO) Cells   The CHO cell line was obtained from ATCC and routinely maintained in HAM'S F-12 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (w/v) FBS. For the collection of recombinant PLP-B, the stably transfected cells were transferred to HAM'S F-12 with 25-40 nM $CdCl_2$, and conditioned media aspirated every 24-48 hr and stored at $-20^{\circ}C$.

Retinal Endothelial Cells   Human capillary endothelial cells were isolated from collagenase-digested donor retinas using the technique described by del Vecchio and Schaffer (1991) and modified as in Grant and Guay (1991). The purity of the primary endothelial cell cultures was evaluated by phase contrast microcopy and immunoflourescent labeling with acetylated low-density lipoprotein labeled with 1,1'dioctadycl-3,3,3',3' tetramethyl-indocarbocyanin perchlorate (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA). Cells from passage 5-7 were used in the migration studies. The primary endothelial cultures were grown on 0.2% (w/v) gelatin coated plates and maintained in DMEM with 10% (w/v) FBS, 0.5 µg/ml insulin and transferrin, 100 µg/ml streptomycin, 100 U/ml penicillin, 0.5 µg/ml Amphotericin B and 0.15 µg/ml endothelial cell growth supplement.

Expression and Purification of the hCAII-PLP-C Fusion Protein

Plasmid p0304/PLP-C23 was transformed into the bacterial expression host JM109(DE3) according to the procedure of Hanahan (1993). Cultures containing p0304/PLP-C23 were grown at 37°C overnight in Luria Broth supplemented with ampicillin (50 μg/ml). The overnight culture was diluted 1:500 in fresh media and grown at 37°C until the optical density measured at 550 nm reached 0.6-0.8. Expression of the fusion protein was induced by adding 0.1 mM isopropyl β–thiogalactosidase and 12.5 μM ZnCl$_2$. Following a 5 hr incubation at room temperature, cells were harvested by centrifugation at 1500 x g for 15 min and the cell pellet was stored at -80°C.

Cells were lysed by freeze-thawing and sonication and resuspended in cold 50 mM Tris/0.5 mM EDTA (pH 7.8) containing 0.1mM PMSF and 25 μM ZnCl$_2$. The suspension was incubated with *DNase*I for 1 hr and then centrifuged for 30 min at 15000 x g . The supernatant was adjusted to pH 8.7 and incubated with 3 ml pAMBS agarose resin overnight. pAMBS affinity chromatography utilizes hCAII as the purification ligand. The resin was recovered by centrifugation and washed with 30 bed volumes of 0.1 M Tris/0.2 M K$_2$SO$_4$/0.5 mM EDTA (pH 9.0) followed by the same buffer at pH 7.0 until the absorbance at 260 and 280 nm was effectively zero. The hCAII-PLP-C fusion protein was eluted from the resin with 0.4 M KSCN/0.1 M Tris/0.5 mM EDTA (pH 6.8), and concentrated using centriprep-10 concentrators (Amicon). The retentate was dialysed for 3-4 days against 1000 volumes of 50 mM Tris/2 mM Ca^{2+} (pH 8). All purification steps were performed at 4°C. Protein concentration of all samples was determined by the method of Bradford (1976).

The dialysed protein was digested (1U enterokinase:3 μg protein) at 37°C for 30-36 hr, and the mixture was then incubated with the pAMBS agarose resin overnight. The resin was collected by centrifugation and the supernatant containing recombinant PLP-C was concentrated, dialysed and stored at -20°C.

<u>Construction of the pET22b(+)PLP-C Bacterial Expression System</u>

<u>Expression Vector:</u>   The expression construct was generated by modification of the original hCAII-PLP-C fusion construct. The hCAII-PLP-C plasmid construct was double digested with *EaeI/Hinc*II to generate a 751 bp fragment containing the PLP-C cDNA insert. The bacterial expression vector pET22b(+) was digested with *NdeI/Not*I. Both fragments were gel purified on 1% (w/v) agarose and the bands isolated with the Quiaex gel extraction kit (Quiagen, Chatsworth, CA).   Insertion of the PLP-C DNA into the pET22b(+) system with a polyhistidine linker region required that oligonucleotide adaptors containing the polyhistidine sequence be annealed to the 5' end of the PLP-C insert.

Upper Primer: 46 mer   5'- TAT GGG CCA TCA TCA TCA TCA TCA TCA TCA TCA TCA CGT GAG CGG C -3'.   Lower Primer: 44 mer 5'-   GCC   GCT CAC GTG ATG ATG ATG ATG ATG ATG ATG ATG GCC CA -3'. To facilitate this procedure a three way ligation reaction was set up with the 750 bp PLP-C insert, pET22b(+) and the oligo adaptors in the presence of T4 DNA ligase overnight at 4°C. Five μl of the ligation reaction was used to transform competent JM109 cells and viable recombinant colonies were screened on LB-Amp plates for the presence of the PLP-C insert using a *Nde*I digestion. A single positive clone was transformed into the bacterial expression host BL21(DE3).

<u>Purification of Recombinant His-PLP-C:</u>   The recombinant bacterial expression system was cultured as previously described for the hCAII-PLP-C fusion protein and stored at -20°C until ready for His-PLP-C purification. The bacterial pellets were lysed in 20 mM Tris, 5 mM Imidazole, 0.5 M NaCl (pH 7.9) containing 0.1 mM PMSF. The suspension was incubated with *DNase*I for 1 hr, and then centrifuged for 30 min at 15000 x *g*. Recombinant His-PLP-C was isolated from the supernatant via the ability of the Histidine tag sequence on the protein to bind to immobilized divalent nickel using the His•Bind® metal chelation resin (Novagen, San Diego, CA). The supernatant was passed over the

nickel column, followed by 30 volumes of wash buffer (60 mM Imidazole, 0.5 mM NaCl, 20 mM Tris pH 7.9). The bound protein was eluted in elution buffer (0.75 M Imidazole, 0.5 M NaCl, 20 mM Tris pH 7.9) and collected in 1ml fractions which were analysed on a UV/Vis spectrophotometer at 280 nm for the presence of protein. Protein fractions were dialysed into 50 mM Tris, 5 mM Ca^{++} (pH 7.9). The dialysed protein was incubated with enterokinase at 37°C overnight, and centrifuged at 1500 x g for 10 min to remove any precipitate. The supernatant containing cleaved recombinant PLP-C was then stored at -20°C.

Amino-terminal Sequence Analysis

N-terminal amino acid sequence was determined by Edman degradation using a gas phase protein sequencer at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) Protein Chemistry Core.

Construction of the PLP-B Mammalian Expression System

Expression Vector: The pGEM3-PLP-B clone (Duckworth et al., 1988) was doubly digested with *Eco*RI/*Hinc*II in order to release the 700 bp cDNA insert containing the complete coding sequence for PLP-B. The digest was run on 1% (w/v) agarose gel and the 700 bp band excised from the gel with a scalpel and purified using the Quiaex gel purification kit (Qiagen, Chatsworth, CA). pMXSND (Lee and Nathans, 1988) was digested with *Xho*I and gel purified. Both cDNA framents were blunt ended by incubation with 2 mM dNTP and 5 U of Klenow at 25°C for 20 min, then the enzyme was heat inactivated by incubation at 75°C for 15 min. pMXSND was further dephosphorylated by incubation with 40 U of Calf Intestinal Alkaline Phosphatase (CIAP) for 90 min at 37°C to inhibit self ligation. Ligation between PLP-B and dephosphorylated pMXSND was set up in a 20 µl volume with 4 U of T4 DNA Ligase at 4°C overnight. Ten µl of the ligation

mixture was used to transform competent JM109 cells. Viable colonies were used to generate minicultures in Luria Broth (LB) and plasmid DNA isolated with the Wizard Miniprep Kit (Promega, Madison, WI). Diagnostic digests were performed by digestion with *Bam*HI to ascertain the presence and correct orientation of the PLP-B insert. Clone 11 possessing the insert in the correct orientation was transformed into competent JM109 cells and a DNA maxiprep performed using a Qiagen Maxiprep kit (Qiagen, Chatsworth, CA) to produce transfection grade plasmid DNA.

<u>Transfection and Stable Mammalian Expression of PLP-B:</u>   The pMXSND-PLP-B construct was transfected into approximately 50-75% confluent CHOK1 cells. Transfection grade plasmid DNA was diluted to 1 mg/ml in ddH$_2$O. Five ml of Lipofectin® was added to 100 µl of OptiMEM for 45 min. pMXSND-PLP-B 1 µg and 2 µg were added to 100 µl of OptiMEM and then mixed with the Lipofectin®. The mixture was allowed to sit for 15 min prior to addition of 1.8 ml of HAM'S F-12. The wells of the plates were washed with serum-free, antibiotic-free media followed by the addition of the Lipofectin-DNA media. Plates were incubated at 37°C and 5% CO$_2$. After 24 hr the media was aspirated and 4 ml of HAM'S F-12, 10% (w/v) FBS added. Cells in each well were trypsinized 48 hr later, diluted 1:100 into G418 selection media (HAM'S F-12, 10% (w/v) FBS supplemented with 800 µg/ml G418) and plated in 10 cm dishes. Colonies were picked using a sterile loop and placed in individual wells of a 96 well plate in selection media. After a week, seven wells were trypsinized and passaged into each of two 10 cm culture plates in selection media. After allowing the cells to attach for 2 days, the cells were fed with methotrexate selection media, (10 µM methotrexate, 10% (w/v) dialysed FBS, 500 µg/ml G418, HAM'S F-12). Cultures were grown to 50-75% confluency and one of each of the two plates treated with 50 nM CdCl$_2$ for 24 hr prior to RNA isolation, to confirm the presence of the message for PLP-B by Northern blot analysis using a ^{32}P-labelled PLP-B cDNA probe. Further selection was performed using methotrexate at concentrations up to 180 µM. Cells were washed in Hank's buffer and incubated in serum-free HAM'S F-12

with 25 nM CdCl$_2$ and conditioned media collected at 24-48 hr intervals and stored at -20°C.

Basal Zone Explant Conditioned Media

Timed pregnant Sprague Dawley rats were obtained from Holtzman (Madison, WI). Animals were housed in a temperature controlled room with 12 hr light/dark cycles and given food and water *ad libitum*. Animals were sacrificed on day 16-19 of gestation under sodium pentobarbital anesthesia. The uteri were removed, and placental basal (junctional) zones were isolated using forceps and dissecting scissors. Basal zone tissue was minced and incubated under sterile conditions in modified Eagle's medium (175 mg tissue/5 ml medium) for 24 hr at 37°C under 47.5% O$_2$:2.5% CO$_2$:50% N$_2$. The culture medium was supplemented with 3 mg/ml glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 0.2 U/ml insulin, 1% v/v non-essential amino acids, 1% v/v MEM vitamins, and 1.5 μg/ml methionine (1/10 normal concentration). After incubation the conditioned medium was centrifuged at 1000 x *g* for 10 min to remove debris and stored at -20°C.

Immuno-Affinity Protein Purification:

The immunoaffinity columns for PLP-B and PLP-C were generated by linking generated polyclonal antipeptide antisera (Ogilvie et al., 1990b) to an activated agarose support matrix Affi-Gel™ (Bio-Rad, Hercules, CA) according to the manufacturer's instruction. For purification of native PLP-B and PLP-C, as well as recombinant PLP-B, conditioned media at neutral pH was passed over the column and the column washed with 30 bed volumes of buffer (0.5 M NaCl, 10 mM phosphate, pH 7.0); bound protein was eluted in 0.2 M Glycine, pH 2.8 and neutralized with K$_2$HPO$_4$, pH 9.2. Neutralized native protein was concentrated using Amicon centriprep concentrators and stored at -20°C until

required. Protein concentrations were estimated by comparison with Coomassie stained BSA standards on SDS-PAGE minigels.

Endothelial Cell Migration Assay

Endothelial cell migration assays were performed essentially as in Grant et al (1987) using modified Boyden Chambers (Neuro Probe Inc., Cabin John, MD). Human retinal endothelial cells were trypsinized from a T75 flask, and pelleted by centrifugation at 500 x g for 5 min at room temperature. The cells were resuspended in Hank's buffer and washed twice. Cells numbers were evaluated on a hemocytometer after staining with 0.25% methylene blue solution. Approximately 7000 cells in 27 μl of serum-free growth media were aliquoted into the lower wells of the migration chamber. The wells were overlaid with a porous bovine dermal collagen coated membrane (5 μm diameter pore size) and the gasket seal and upper chamber attached. The apparatus was inverted at 37°C and 5% CO_2 for 90 min to allow for cellular attachment, after which test protein solutions in serum-free media were added in a total of 50 μl/well in sextuplicate. 10% FBS and serum-free media were used as positive and negative controls, respectively. The apparatus was incubated overnight at 37°C/5% CO_2 to facilitate migration of attached cells. The apparatus was disassembled, backside (cellular attachment side) scraped, and the membrane stained in Leukostat™ stain. The membrane was then mounted on a glass slide for evaluation by counting the numbers of migrated cells/well under a light microscope. For migration assays, cells were evaluated in quadruplicate wells for each treatment regimen.

In Vitro Invasion Assay

The Matrigel invasion assay was performed using the modified Boyden Chamber apparatus as in the endothelial cell migration assay (Grant et al, 1987). RL95-2 cultures

were incubated with B(a)P and TCDD for 48 hr. Cells were trypsinized and pelleted by centrifugation at 500 x g for 5 min. The cells were resuspended in Hank's buffer and washed twice. Cells numbers were determined using a hemocytometer. Approximately 7000 cells in 27 µl of complete media in the presence of the respective chemicals were aliquoted into the lower wells of the Boyden chamber. The wells were overlaid with a porous Matrigel-coated polyvinyl-pyrrolidone-free polycarbonate membrane (8 micron diameter pore size) and the gasket seal and upper chamber attached. The apparatus was inverted at 37°C/5% CO_2 for 90 min to allow for cellular attachment, after which time 50 µl of complete media was added to the upper wells. The apparatus was incubated for 36 hr at 37°C/5% CO_2, at which time the apparatus was disassembled, the backside of the membrane (cellular attachment side) scraped, and the membrane stained in Leukostat™ stain. The membrane was then mounted on a glass slide for evaluation under a light microscope by counting the number of cells per well which had invaded the membrane. For these assays, cells were counted in quadruplicate wells for each treatment regimen.

Nb2 Assay for Lactogenic Activity

The Nb2 proliferation assay was performed essentially as described by Gout et al (1980). The lactogenic response was evaluated using [^{3}H]-thymidine incorporation and MTT dye conversion as indicators of the growth response. Log phase Nb2 cells in suspension were collected by centrifugation, washed, and resuspended in starvation media overnight. Cells were again collected by centrifugation and 4×10^4 cells in 100 µl were added to the triplicate wells on a 96 well plate. Ovine PRL (NIDDK) in concentrations between 0.1-100 ng/ml was added in triplicate wells in 50 µl aliquots as the standard stimulatory response, while native and recombinant PLP-B and PLP-C were added in the same volume. The cultures were harvested onto microfiber filter paper after a 4 hr [^{3}H]-thymidine pulse using a Brandel cell harvester at 48 hr (Gaithesburg, MD), and [^{3}H]-

thymidine uptake was measured using a Beckman LS 7000 liquid scintillation counter. Alternatively, Nb2 cells and the formazan product were solubilized at 48 hr after a 4 hr exposure to MTT, and the absorbance measured at 595 nm on an Elisa plate reader (Adler et al., 1994).

EGFR Binding Assay

RL95-2 cells were grown for 24-96 hr in monolayer culture in the presence of 0-50 nM TCDD in serum-free medium or in the presence of 2% (w/v) or 10% (w/v) FBS supplemented DMEM/HAM'S F-12. Cells were washed three (3) times with cold PBS (pH7.4), scraped from plates with a rubber policeman and pelleted by centrifugation. Fifty μg of protein (whole cells) was incubated in serum-free DMEM-HAM'S F-12 containing 0.1% (w/v) BSA, with 400 pg [^{125}I]-EGF (8 x 10^4 cpm) in the presence or absence of 100 ng unlabelled EGF for 16 hr at 4°C. Incubations were stopped and unbound radioactivity removed by washing cells twice in cold PBS. Total bound cpm was measured using a gamma counter. Specific binding was expressed as the difference between radioactivity bound in the absence (total) and presence (non-specific binding) of excess unlabelled EGF.

Western Immunoblot Analysis

General Procedure: Cells were rinsed two to three times, collected with a cell scraper, and lysed in 0.5-1 ml PBS using three freeze-thaw cycles. The membrane fraction was obtained after centrifugation at 10000 x g for 15 min at 4°C and then resuspended in ddH$_2$O. Alternatively, cells were scraped into lysis buffer containing 10 mM Tris, 0.14 M NaCl, 0.5% (w/v) Na-deoxycholate, 1% (v/v) Triton-X 100, 1 mM phenyl-methyl sulfonyl fluoride (PMSF), and 1 μg/ml each of leupeptin and aprotinin. Cells were then incubated with the lysis buffer for 1 hr at 4°C on an orbital shaker and the supernatant was recovered after centrifugation at 10000 x g for 15 min at 4°C. Samples of membrane or total cell lysate protein (50-100 μg) were then separated by 7.5% or 10% SDS-PA gels and

transferred electrophoretically to nitrocellulose filters using 25 mM Tris, 192 mM glycine buffer at pH 8.2, with 20% (v/v) methanol according to the method of Towbin et al (1979).

EGF Receptor and CYP1A1 Protein: For EGF receptor and CYP1A1 protein analysis, immunostaining was performed as previously described in Wang et al (1987).   The nitrocellulose membranes were washed in 20 mM Tris containing 0.1% (w/v) Tween 20 and 0.9% (w/v) NaCl, pH 7.5 (TTBS) for 15 min, and then 3% (w/v) gelatin for 30 min. The membrane was then sequentially incubated with polyclonal anti-human EGF receptor antiserum diluted to 1 µg/25ml in TTBS without Tween 20 or preimmune sheep serum for 2 hr, followed by horseradish peroxidase labeled goat anti-sheep IgG for 60 min. Alternatively, for CYP1A1 the membrane was incubated with polyclonal goat anti-rat CYP1A1 (1:1000 dilution) or preimmune goat serum for 2 hr, followed by horseradish peroxidase conjugated anti-goat IgG for 1 hr. Bands were visualized by incubation with 3-amino-9-ethylcarbazole in the presence of 0.015% (v/v) hydrogen peroxide. Immunoreactive bands were quantitated by scanning the nitrocellulose filters on a Microtek ScanMaker II scanner and NIH image software.

PLP-B and PLP-C Protein: The blotted nitrocellulose membranes were blocked in 3% gelatin for 30 min.   The membrane was then sequentially incubated with either polyclonal rabbit anti-rat PLP-B or PLP-C antiserum diluted 1:1000 in TBS or preimmune rabbit serum for 2 hr. This was followed by incubation with horseradish peroxidase labeled goat anti-rabbit IgG for 60 min.   Immunoreactive bands were visualized by incubation with 3-amino-9-ethylcarbazole in the presence of 0.015% hydrogen peroxide.   Immunoreactive bands were quantitated as described above.

RNA Isolation & Northern Blot Analysis

Total cellular RNA was isolated from TCDD-treated cell cultures by acid guanidium thiocyanate phenol-chloroform extraction according to Xie and Rothblum (1991).   For Northern blotting, 40 µg of total cellular was denatured, fractionated on a 1.5% (w/v)

agarose formaldehyde gel, and transferred to nylon membranes. cDNA probes were labelled with [α-^{32}P]-dCTP using a random primer labelling kit. Prehybridization was carried out in commercially available ExpressHyb buffer at 68°C for 1 hr, and hybridization in the same buffer for 2-4 hr after addition of the ^{32}P-labelled probe. The membranes were washed three times in 2 X SSC, 0.1% (w/v) SDS at room temperature for 10 min, then twice in 0.1 X SSC, 0.1% (w/v) SDS at 60-65°C for 30 min. Transcripts were then visualized by autoradiography. Blots were later stripped and rehybridized with ^{32}P-labelled cDNA for β-actin as a loading and transfer control in order to facilitate quantitation by optical scanning.

<u>Nuclear Run Off Assay</u>

Nuclei were prepared by a detergent lysis protocol (Ausubel et al., 1987). Actively proliferating cultures of RL95-2 cells were exposed to 10 nM TCDD or 0.1% (v/v) DMSO for 40 hr, after which the cells were washed twice with cold PBS, scraped from the plates with a rubber policeman and pelleted by centrifugation at 500 x g for 5 min. The cell pellet was resuspended by vortexing in 4 ml of NP-40 lysis buffer [10 mM Tris, 10 mM NaCl, 3 mM MgCl$_2$, 0.75% (v/v) NP-40 pH 7.4], placed on ice for 5 min, after which nuclei were collected following a second round of centrifugation at 500 x g. This process was repeated and nuclei resuspended in 500 μl of glycerol storage buffer [50 mM Tris, 5 mM MgCl$_2$, 0.1 mM EDTA, 40% (v/v) glycerol, pH 8.3] per 10^8 nuclei. Nuclei were stored at -80°C in 200 μl aliquots until ready for use.

Nuclear runoff assays were performed essentially according to Srivastava et al (1993) and Merscher et al (1993) with minor changes. Frozen nuclei ($\approx$ 4 x 10^7) per reaction were thawed and mixed with an equal volume of 2 X reaction buffer (10 mM Tris, 5 mM MgCl$_2$, 0.3 M KCl, 5 mM DTT, 0.2 mM EDTA, 4 mM ATP, GTP, CTP) and 75 μCi [α-^{32}P]UTP. The reaction was allowed to proceed in a room temperature water bath for 30 min at which time 50 U $DNase$I was added and the reaction allowed to continue for

5 min. After labelling, the nuclear suspension was mixed with 1.2 ml of guanidium isothiocyanate reagent (Xie and Rothblum, 1991), 130 µl chloroform- isoamylalcohol (24:1), and vortexed. The reaction tube was left on ice for 10 min and the aqueous layer collected after centrifugation at 10000 x g for 10 min and incubated with an equal volume of isopropanol at -80°C overnight. The following day the RNA pellet was collected after centrifugation at 10000 x g for 10 min and the pellet washed once in 70% (v/v) ethanol. The pellet was air dried and dissolved in 0.5% SDS and 1 µl used to determine activity using a scintillation counter. Equal amounts of cpms ($\approx 10^6$) from DMSO control and TCDD experiments were added to 2 ml of ExpressHyb buffer and hybridized with appropriate cDNAs on nylon membranes at 65°C for 48 hr. Membranes were then washed in 2 X SSC at room temperature for 30 min, then by 0.1 X SSC, 0.1% (w/v) SDS at 62°C for 1 hr. Dot blots were visualized by autoradiography following 2-5 day exposure at -80°C. X-ray films were scanned using a Microtek ScanMaker II scanner and spot intensities quantitated by densitometry with each message standardized against β-actin as control.

Fibrin Zymography

Fibrin zymography was performed according to the method of Granalli-Piperno and Reich (1978) with modifications by Cheng et al (1991) and Nakamura et al (1995). Briefly, RL95-2 cells were seeded into 10 cm plates at 5 x 10^5 cells per plate and allowed to grow for three days. Cultures were exposed to TCDD and B(a)P for 36 hr and the cultures washed in Hank's buffer before the addition of equal volumes of serum-free media to each plate. Conditioned media was collected from control and treated cultures after 12 or 24 hr. Thirty µl of CM was mixed with SDS sample buffer and run under non-reducing conditions on a 10% SDS-PA gel. Gels were then immersed in 50 mM Tris, 5 mM CaCl$_2$, 2.5% (v/v) Triton X-100 three times for 15 min to remove the SDS, and then twice more (50 mM Tris, 5 mM CaCl$_2$, pH 8.1) for 15 min to remove the Triton X-100. To detect the

plasminogen activator activity, the polyacrylamide gel was overlaid onto a 1% (w/v) low temperature gelling agarose gel containing 75 µl of 1mg/ml of plasminogen, 30 µl of 1 U/ml thrombin, and 10 ml of 5 mg/ml fibrinogen, in phosphate buffered saline (PBS). The gels were incubated overnight in a humidified atmosphere at room temperature. Plasminogen activator activity was detected as a lysed zone on the agarose gel after staining with Coomassie brilliant blue or amido black. Bands were scanned and fibrinolytic activities assessed by comparison of the areas of the lytic zones.

Data Analysis

All experiments were performed at least three times at concentrations and time points indicated unless otherwise stated. For analysis of scanned images, control lanes were standardized to 100% and all treatments assessed relative to controls for each indiviual experiment. For Northern blots loading was standardized to β-actin. One factor ANOVA analysis was performed to assess the dose-response effects with significance being determined at the $p < 0.05$ level. Numerical data averaged over several experiments was represented as Mean ± Standard Error of the Mean (SEM) and the Student's t-test used to analyse the data. Statistical analyses were performed using Microsoft Excel software program.

EFFECTS OF TCDD AND B(a)P ON CELLULAR PROLIFERATION AND EGF
RECEPTOR EXPRESSION IN THE RL95-2 CELL LINE

Introduction

Endometriosis is the ectopic growth of endometrial tissue outside of the uterus and has been described as a disorder which has characteristics of both a benign disease and an invasive neoplasm (Arnold et al., 1996). It is first and foremost a proliferative disorder in which endometriotic tissue is mislocated to and invades extrauterine sites. In view of recent evidence implicating environmental agents like TCDD and cigarette smoking in the etiology of endometriosis (Cummings et al., 1996; Mattorras et al., 1995; Mayani et al., 1997; Rier et al., 1993), our laboratory undertook an investigation as to whether or not these environmental agents are able to directly alter the cellular proliferation of endometrial cells.

Human uterine endometrium proliferates in response to estrogen during the course of the female menstrual cycle. The growth and maintenance of endometriotic tissue is known to be dependent upon estrogen as evidenced by its extreme rarity in premenarchal girls and the association with exogenous estrogen administration in postmenopausal women (Goodman et al., 1989). Recent evidence suggests that the action of estrogen on the endometrium is mediated by EGF, with EGF being able to mimic the stimulatory effects of estrogen on DNA synthesis and lactoferrin gene expression in ovariectomized mice (Nelson et al., 1991). Estrogen also increases EGF binding and mRNA levels for the EGF receptor in uterine tissue (Lingham et al., 1988; Mukku and Stancel, 1985).

TCDD and B(a)P have been shown to generally downregulate EGF receptor expression in different tissues and cell lines (Astroff et al., 1990; Guyda et al., 1990; Hudson et al., 1986; Sewall et al., 1993; Zhang et al., 1995), although upregulation of EGF expression by TCDD has been demonstrated developmentally (Abbott and Birnbaum, 1990; Abbott et al., 1992). EGF receptor expression has been demonstrated in human endometriotic tissue (Huang and Yeh, 1994; Prentice et al., 1992), as well as in endometriotic lesions from surgically-induced animal models (Simms et al., 1991; Zhang et al., 1993a). Therefore, EGF receptor expression may have a role to play in the pathogenesis of endometriosis.

Our study evaluated the potential changes in EGF binding and EGF receptor protein expression in endometrial cells subsequent to TCDD and B(a)P exposure. The goal of the study was to ascertain whether or not a potential correlation could be made between EGF receptor expression and cellular proliferation, as well as with the induction of the metabolic enzymes CYP1A1 which is classically induced by TCDD and B(a)P exposure (Sutter et al., 1994; Whitlock, 1989).

Results

Effects of TCDD and B(a)P on CYP1A1 and CYP1B1 mRNA in RL95-2 Cells

We initially examined the ability of TCDD and B(a)P to induce the expression of mRNA for CYP1A1, a classic functional biomarker of exposure to AhR agonists (Whitlock, 1989). Northern blot analysis of 0.1% (v/v) DMSO (control) treated RL95-2 cells indicated that the 3.0 kb mRNA transcript for CYP1A1 was virtually undetectable. However, 48 hr exposure to TCDD at a concentration of 0.1 nM TCDD was able to significantly induce CYP1A1 mRNA expression with induction being maximal at the 1 nM TCDD level (Figure 3-1A). In data not shown, induction of CYP1A1 mRNA by 10 nM TCDD was observed as early as 6 hr after exposure. Similarly, 10 µM B(a)P exposure for

12 hr resulted in significant CYP1A1 mRNA induction, with levels being maximal by 24 hr and maintained for the 48 hr assay period (Figure 3-1B). The expression of CYP1B1 mRNA, a second drug metabolizing enzyme recently shown to be induced by TCDD (Sutter et al., 1994), was further analyzed by Northern blot analysis. As shown in Figure 3-1A, RL95-2 cultures express a constituitively low level of the 5.1 kb CYP1B1 mRNA transcript which is readily induced by TCDD concentrations as low as 0.1 nM. Hence the RL95-2 cell line appears to possess a functional AhR signal transduction system as described in Chapter one.

Effects of TCDD on ^{125}I-EGF Binding in RL95-2 Cells

The ability of TCDD to alter the total specific binding of ^{125}I-EGF to intact RL95-2 cultures was evaluated. Cultures treated with 1-100 nM TCDD for up to 96 hr did not exhibit any significant change in the total binding of ^{125}I-EGF as compared to 0.1% (v/v) DMSO-treated controls (Figure 3-2). Total specific ^{125}I-EGF binding was in the range of 10-12% as has been previously demonstrated for this cell line (Korc et al., 1986). In this regard, it is pertinent to note that these experiments were performed at 4°C so as to minimize internalization of the transmembrane EGF receptor.

Effects of TCDD and B(a)P on EGFR and CYP1A1 Protein in RL95-2 Cells

Exposure of RL95-2 cultures to 1 nM and 10 nM TCDD showed a marked induction of the 55 kDa CYP1A1 protein band as determined by Western immunoblot analysis (Figure 3-3A). In data not shown, this induction of immunoreactive protein was shown to be concentration-dependent being maximal at 1 nM TCDD. Similarly, exposure to both 1 μM and 20 μM B(a)P also resulted in a dose-dependent induction of CYP1A1 immunoreactive protein, although the level of induction was not as high as that exhibited by TCDD treatment (Figure 3-3A). The induction of immunoreactive protein correlated with the observed induction of mRNA transcripts for CYP1A1 after exposure of RL95-2

cultures to both agents (Figure 3-1A & B). The levels of the 170 kDa immunoreactive EGF receptor membrane protein band were unchanged after TCDD exposure at concentrations of 1 nM and 10 nM relative to 0.1% (v/v) DMSO controls (Figure 3-3A & B). In data not shown, no effect of TCDD was noted for exposures up to 96 hr. By contrast, 20 μM B(a)P almost totally eliminated the expression of EGFR immunoreactive membrane protein after a 48 hr exposure period (Figure 3-3A & B). This decrease in EGFR immunoreactive protein levels is an effect associated with B(a)P which has been previously observed in placental choriocarcinoma cells (Zhang et al., 1995). Thus the decrease in EGFR immunoreactive protein with B(a)P exposure was correlated with a corresponding induction in CYP1A1 protein in this cell line. In contrast, TCDD treatment exhibited no observable decreases in EGFR protein levels while significantly greater CYP1A1 induction was observed.

Effects of TCDD and B(a)P on Cellular Proliferation in RL95-2 Cells

The effects of TCDD and B(a)P on RL95-2 cell proliferation were evaluated by the direct counting of viable cells. Under serum-free conditions, 10 μM B(a)P significantly decreased the rate og growth of RL95-2 cells by 48 hr after exposure (Figure 3-4). Similarly, for cultures growing in medium containing 10% (v/v) FBS, the number of cells in 10 μM B(a)P-treated cultures was lower than those of 0.1% (v/v) DMSO controls by as early as 24 hr (Figure 3-5). Furthermore, under serum-free conditions the number of viable cells in B(a)P treated cultures appeared to plateau after 48 hr with no further increase in cell numbers being observed for the remaining 24 hr of the assay. In contrast, 10 nM TCDD did not result in any significant change in cell numbers compared to control cultures for the duration of the 72 hr assay period. Thus 10 μM B(a)P, but not 10 nM TCDD, adversely affected cellular proliferation of RL95-2 cells under both serum-free and complete media conditions.

<u>Effect of TCDD and B(a)P on Steady State *c-myc* mRNA Levels</u>

Northern blot analysis was next used to determine whether TCDD and B(a)P exposure of RL95-2 cultures could lead to alterations in the expression of the steady state levels of *c-myc* mRNA, a proto-oncogene associated with cellular proliferation (Västrik et al., 1994). Data in Figure 3-6 show the presence of a strong constitutive level of expression for the 2.7 kb mRNA transcript in control cells. Expression was not significantly altered by up to 48 hr exposure to either 10 nM TCDD or 10 μM B(a)P (Figure 3-6A & B). Hence the decreased ability of RL95-2 cells to proliferate in the presence of B(a)P exposure is not correlated with a decreased level of *c-myc* mRNA expression.

<u>Discussion</u>

We chose to investigate the potential alterations in EGF receptor expression and cellular proliferation after treatment with TCDD and B(a)P in order to gain a better understanding of the effect these environmental agents might have on uterine growth and uterine disease pathologies. Endometriosis is an estrogen-dependent disease (Barbieri, 1990) being rare before puberty or after menopause. Recent data indicate that estrogen actions on the uterus may be mediated through growth factors like EGF as well as increases in EGF receptor expression (Lingham et al., 1988; McBean et al., 1997; Nelson et al., 1991). EGF has also been shown to be mitogenic in human endometriotic stromal tissue (Mellor and Thomas, 1994), while experiments with rat endometriosis models suggest that rat endometrial implants produce EGF and contain receptors for EGF (Simms et al., 1991). Furthermore, Danazol or gonadotropin releasing hormone analogues (GnRHa), clinical therapies for relieving the symptoms of endometriosis, significantly decrease the levels of immunohistochemical staining for the EGF receptor (Melega et al., 1991).

The RL95-2 cell line was found to express high levels of the 170 kDa immunoreactive EGF receptor protein in agreement with previous reports (Korc et al., 1986, 1987; Lelle et al., 1993). In data not shown, these cells also exhibited the presence of the 5.6 kb mRNA transcript for the EGF receptor as has been demonstrated in other systems (Lin et al., 1991; Zhang et al., 1995). The specific binding of ^{125}I-EGF was not altered by exposure to concentrations of TCDD up to 100 nM for the duration of the 96 hr assay period compared to vehicle treated controls. This result was supported by Western immunoblot analysis for EGF receptor, while the levels of the 5.6 kb mRNA transcript for EGF receptor were also not significantly altered by TCDD treatment (data not shown). In contrast to TCDD, exposure of RL95-2 cultures to B(a)P resulted in a concentration-related decrease in the levels of the 170 kDa immunoreactive EGF receptor protein as determined by Western analysis.

Tissue specific effects of TCDD and B(a)P on EGF receptor expression and binding has been a well characterized toxicological observation (Pohjanvirta and Tuomisto, 1994). TCDD has been demonstrated to decrease the binding capacity of EGF receptors in the liver (Sewall et al., 1995), uterine tissue (Astroff et al., 1990) and in hepatoma cells and keratinocytes (Hudson et al., 1985, 1986; Kärenlampi et al., 1983) and, in some cases, without a consequent decrease in steady state EGF receptor mRNA levels (Lin et al., 1991). It should be noted that TCDD has been found to increase EGF receptor expression during early development in the embryonic mouse palate and ureteral epithelium (Abbott and Birnbaum, 1990; Abbott et al., 1992). In contrast, B(a)P has exhibited a similar effect in downregulating EGF receptor protein levels in human keratinocyte and placental cell lines (Guyda et al., 1990; Hudson et al., 1985; Zhang et al., 1995).

The mechanism by which these compounds are able to alter EGF receptor binding and expression are not well understood. Evidence that the effects of TCDD on the hepatic EGF receptor may be mediated via the *Ah* locus was found in congenic strains of mice differing at this locus (Lin et al., 1991). In this regard, prolonged exposure in rats

demonstrated that the ED_{50} for the decrease in receptor capacity was close to the ED_{50} for CYP1A1 induction, which is known to be regulated transcriptionally by TCDD (Sewall et al., 1993). Furthermore, the inhibition of EGF-specific binding has been shown to be stereospecific in that a TCDD analogue unable to bind to the Ah receptor was not able to decrease EGF binding (Hudson et al., 1985). The results of our study indicate, however, that both TCDD and B(a)P were able to induce the expression of both CYP1A1 mRNA and immunoreactive protein levels in the RL95-2 cell line, while only B(a)P was able to selectively decrease EGF receptor expression.

Kärenlampi et al (1983) have proposed that electrophilic metabolites of polycyclic aromatic hydrocarbons may be responsible for the inhibition of EGF binding observed in mouse hepatoma cells. In this regard, TCDD is not readily metabolized and has a significant 7-10 year half-life in humans, while B(a)P is readily metabolized to a series of reactive species including epoxides, phenols and quinones (Gelbion, 1980). Thus the observed down-modulation of EGF receptor protein by B(a)P may be a direct effect of reactive metabolites which would not be observed with TCDD due to its resistance to metabolism. Alternatively, EGF receptor down-modulation can be effected by changes in cytokines such as IL-1 and TNF (Bird and Saklatvala, 1990). As will be seen in Chapter Five, however, TCDD is able to induce the expression of these cytokines in RL95-2 cells without the observed decrease in EGF receptor protein.

Uterine endometrium is highly responsive to estrogen action. Evidence that the in vivo administration of estrogen was able to stimulate *c-myc* expression at the transcriptional level in rat uterus has led to the suggestion that *c-myc* expression is related to estrogen-induced uterine cell proliferation (Murphy et al., 1987). Similarly, exposure of RL95-2 cell cultures to estrogen has been demonstrated to induce the expression of *c-myc* in a time-dependent manner (Liu and Teng, 1994). In general, exposure of cells to estrogen in vitro has not been demonstrated to be mitogenic in isolated uterine cell cultures (Tomooka et al., 1986), nor to induce *c-myc* mRNA in primary endometrial epithelial cells (Jouvenot et al.,

1990). Withdrawal of cells from the cell cycle by removal of growth factors leads to down-regulation of *c-myc* expression and may be a requirement for growth arrest (Waters et al., 1991). Furthermore, deregulated *c-myc* expression can induce apoptosis in fibroblasts deprived of growth factors (Evan et al., 1992), suggesting that this protein may serve to integrate the effects of different signaling pathways for cellular growth.

Endometriosis is characterized by the extrauterine proliferation of endometrial tissue. Characterization of the effects of TCDD and B(a)P on proliferation of endometrial cell cultures would provide a better understanding of the potential contribution of these xenobiotics to the etiology of uterine disease. The present study indicates that B(a)P, but not TCDD, was able to significantly decrease the proliferative ability of RL95-2 cells in culture under both serum-free and complete growth media conditions. In fact B(a)P treatment resulted in virtual arrest of the growth of RL95-2 cells as seen in the plateau in cell numbers after B(a)P administration. In contrast, neither TCDD nor B(a)P were able to alter the expression of the steady state levels of mRNA for c-myc. Therefore the inhibition of cellular growth mediated by B(a)P does not appear to have a direct causal relationship with *c-myc* mRNA levels in this cell line.

In summary, B(a)P, but not TCDD, is able to significantly decrease the expression of immunoreactive EGF receptor protein in RL95-2 cell cultures, and there does not appear to be a direct relationship with the ability of these agents to induce the expression of CYP1A1 and 1B1, members of the cytochrome P450 family. It is possible that reactive metabolites of B(a)P have a role to play in the process of EGF receptor down-modulation. These data correlate with the etiological association of TCDD with enhanced and B(a)P with decreased incidences of endometriotic lesions, respectively, insofar as previously published data has shown no significant differences in EGF receptor expression between normal and ectopic endometrium (Huang and Yeh, 1994; Prentice et al., 1992). Furthermore, B(a)P, but not TCDD, is able to inhibit the cellular proliferation of RL95-2 cultures without a concomitant change in the expression of *c-myc* mRNA levels. Since *c-*

myc expression may not be directly related to the proliferative phenotype, but indirectly involved in the process, a direct correlation may not be observable with *c-myc* mRNA levels.
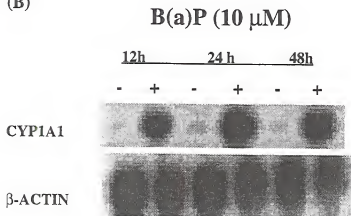
(A)

(B)

B(a)P (10 μM)
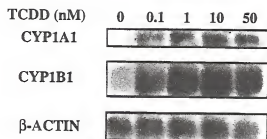
Figure 3-1. Northern blot analysis of the effect of TCDD and B(a)P on the CYP1A1 and CYP1B1 expression in RL95-2 cells. Total RNA was denatured, blotted, and hybridized with ^{32}P-labeled cDNA probes for CYP1A1 and CYP1B1. (A) Dose-dependent induction of CYP1A1 and CYP1B1 by TCDD upon a 48 hr exposure to TCDD. (B) Time-dependent induction of CYP1A1 mRNA after exposure to 10 μM B(a)P.
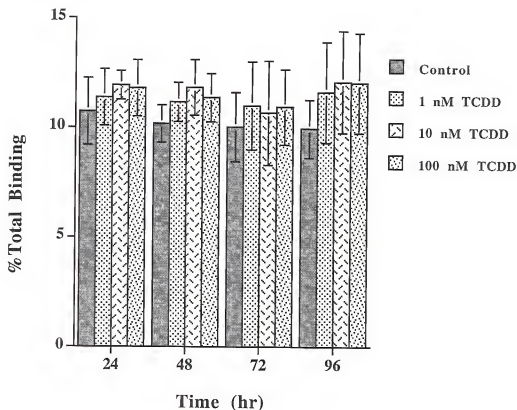
Figure 3-2. Graphical representation of the specific binding of [^{125}I]-EGF to RL95-2 cells after treatment with 0-100 nM TCDD. Cells were incubated with TCDD in the presence of 10% FBS over a 96 hr period. Cellular protein, 50 μg, was incubated with 400 pg [^{125}I]-EGF in the presence and absence of excess (100 ng) unlabeled EGF. Data represent the mean ± SEM of at least three separate determinations .
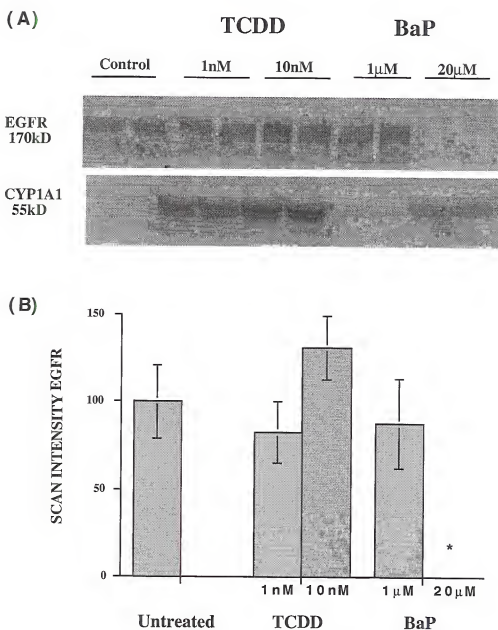
(A)

(B)

Figure 3-3. Effect of TCDD and B(a)P on EGFR immunoreactive protein levels in RL95-2 treated cultures. Actively growing cultures were treated with TCDD or B(a)P for 48 hr and total cell lysate run on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose and probed with sheep anti-human EGF receptor antiserum. (A) Western blots of representative experiments. (B) Quantitation of changes in the level of EGF receptor protein in cultures treated with TCDD and B(a)P. Data are the mean ± SE for three experiments. * P < 0.001 compared to controls.

Figure 3-4. Quantitation of the effect of TCDD and B(a)P exposure on the proliferation of cultures of RL95-2 cells in serum-free media. Actively growing cultures were put into serum-free media in the presence of 0.1% (v/v) DMSO, 10 nM TCDD, or 10 μM B(a)P as described in Materials and Methods. After designated times points cells were trypsinized and counted under a light microscope. Points represent mean ± SEM from three separate experiments. * P < 0.01 compared to control.
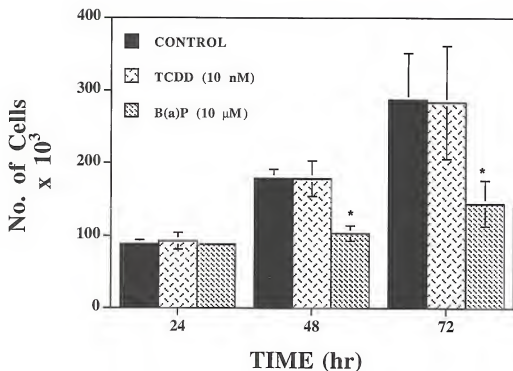
Figure 3-5. Quantitation of the effect of TCDD and B(a)P exposure on the proliferation of cultures of RL95-2 cells in complete media. Actively growing cultures were put into complete media in the presence of 0.1% DMSO, 10 nM TCDD, or 10 μM B(a)P as described in Materials and Methods. After designated times points cells were trypsinized and counted under a light microscope. Points represent mean ± SEM from three separate experiments. * P < 0.01 compared to control.
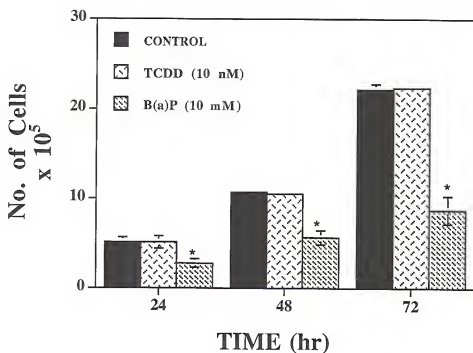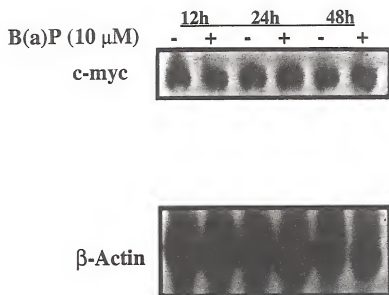
(A)

B(a)P (10 µM)

(B)

TCDD (10 nM)
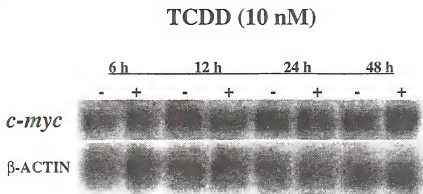

Figure 3-6. Representative Northern blot analysis of c-myc mRNA. Cells were treated with TCDD or B(a)P up to a 48 hr time point and total RNA was isolated, denatured, blotted and hybridized with ^{32}P-labeled cDNA probe for c-myc as described in Materials and Methods. Representative Northern blot for (A) 10 µM B(a)P exposure and (B) 10 nM TCDD treatment of RL95-2 cultures.

CHAPTER 4
EFFECTS OF TCDD AND B(a)P ON CELLULAR INVASIVENESS AND THE
EXPRESSION OF uPA AND TIMPs IN A HUMAN ENDOMETRIAL CELL LINE
RL95-2

Introduction

In endometriosis, uterine endometrial tissue is thought to mislocate to and invade extrauterine sites as a result of retrograde menstruation (Sampson, 1927). The growth regulation of endometriotic tissue, however, is poorly understood since a majority of women of reproductive age women exhibit retrograde flow, yet only around 10% of them will manifest symptoms of the disease, (Olive and Schwartz, 1993). This suggests that those women who develop endometriosis have endometriotic tissue which is more prone to implant and invade the peritoneum, possibly through the action of directed and localized extracellular proteolysis, which may involve plasminogen activators (PA) (Fernández-Shaw et al., 1995). Alternatively, endometriosis is also associated with the presence of adhesions in the peritoneal cavity which has led to the suggestion that patients with endometriosis might have a deficiency in PA activity in the peritoneal fluid, resulting in more permanent adhesions because of reduced fibrin clearance (Malick, 1982).

The process of cellular implantation is highly regulated because dysregulation may have profoundly undesirable consequences, as manifested in tumor metastasis. Figure 4-1 presents a simplified schematic of potential actors in the process of peritoneal implantation based on the Sampson hypothesis (Edwards et al 1996; Stetler-Stevenson et al 1993). Urokinase PA (uPA), a serine protease, may play a role in cellular invasion by directly acting to degrade extra-cellular matrix (ECM) proteins such as fibronectin (Quigley et al., 1987). In addition, uPA can also act through its ability to cleave plasminogen to plasmin,

a protease which is capable of cleaving other ECM proteins as well as activating members of the family of enzymes known as matrix metalloproteinases (MMPs) (see Mayer 1990; Mignatti and Rifkin, 1993 for reviews).

MMPs are a family of enzymes involved in ECM turnover and tissue remodeling (Stetler-Stevenson et al., 1993). Their activity can in turn be modulated by interaction with specific proteinase inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), of which four have now been characterized (Boone et al., 1990; Docherty et al., 1985; Greene et al., 1996; Hammani et al., 1996; Silbiger et al., 1994; Stetler-Stevenson et al., 1990). In the case of MMPs, active enzymes are inhibited by interaction with TIMPs which form tight-binding 1:1 complexes with the MMP enzyme active sites (Stetler-Stevenson et al., 1993). An imbalance between proteinases and their activators and inhibitors can be implicated in a number of pathological states including tumor invasion (Liotta and Stetler-Stevenson 1991).

The goal of this study was to determine whether TCDD and B(a)P could alter the invasive properties of endometrial cells as a potential etiological factor in uterine disease. TCDD has recently been demonstrated to induce the expression of uPA in human keratinocyte cell lines (Gaido and Maness, 1994, 1995). Our study thus evaluated the effect of TCDD and B(a)P on the ability of RL95-2 cells to invade matrigel membranes. The effect of these xenobiotics on the levels of uPA mRNA was also determined along with measurement of potential changes in the fibrinolytic activity of endometrial cells exposed to these agents. Finally, changes in the mRNA levels for TIMP 1 and 2 were also examined in order to determine whether these xenobiotic agents are able to up- or down-modulate other components of the tissue remodeling network.

Results

Evaluation of the Effect of TCDD and B(a)P on RL95-2 Cellular Attachment and
Invasiveness

The ability of endometrial cells to invade tissue at extrauterine sites is one of the
requirements of the Sampson hypothesis (Sampson 1927). The ability of RL95-2
endometrial cultures to invade matrigel membranes after TCDD and B(a)P exposure was
evaluated utilizing a modified Boyden Chamber apparatus (Figure 4-2). The results are
represented graphically in Figure 4-3 and indicate that 10 μM B(a)P, but not 10 nM
TCDD, was able to significantly inhibit the invasion of matrigel membranes as compared
to control cultures ($p < 0.001$). The numbers of invasive cells was decreased in
comparison to the control by up to 95% following a 48 hr exposure of cultures to 10 μM
B(a)P (Figure 4-3). Cellular invasion is a multi-faceted process involving cellular
attachment and chemotaxis, as well as the degradation of basement membrane
components.

In order to ascertain whether or not the observed alteration in cellular invasiveness
of B(a)P treated RL95-2 cultures was associated with cellular attachment, cells were
allowed to attach to matrigel membranes for up to 24 hr. The numbers of attached cells
were evaluated using a light microscope after nuclear staining. The results of a 2 hr
attachment protocol are shown in Figure 4-4A & B. Forty-eight hr exposure to 10 μM
B(a)P resulted in a significantly decreased ability of RL95-2 cells to attach to a matrigel
membrane compared to DMSO treated controls ($p < 0.001$). This result was reproducible
even for attachment periods of up to 24 hr (data not shown). It should be noted that the
cells used in the attachment and invasion assays exhibited greater than 95% viability as
determined by tryphan blue dye exclusion.

Effect of TCDD and B(a)P on uPA mRNA Steady State Levels and Plasminogen Activity in RL95-2 Cells

Northern blot analysis of the 2.8 kb mRNA transcript for uPA in RL95-2 cultures indicated that a high level of constitutive expression exists in control cells. Exposure of cultures to 10 nM TCDD, but not 10 μM B(a)P, resulted in a 2-fold, time-dependent induction of uPA mRNA steady state levels (Figure 4-5A). The observed increase in steady state message for uPA mRNA was significant by 36 hr and remained elevated for the duration of the 48 hr assay period (Figure 4-6B). Furthermore, this increase was also dose-dependent, with significant induction not being observed below the 10 nM TCDD exposure level (Figure 4-6A & B). In contrast, neither 1 μM nor 10 μM B(a)P exhibited any significant alteration in uPA mRNA levels after a 48 hr exposure of RL95-2 cells (Figure 4-6B).

We next chose to investigate whether the level of fibrinolytic activity in the conditioned media from cultures exposed to TCDD and B(a)P was subsequently altered. Fibrin zymography is a classic assay for biological activity of uPA (Figure 4-7A) which relies upon the ability of PA within the lanes on an SDS-PA gel to act upon plasminogen to generate active plasmin and hence produce fibrin degradation products which show up as lytic zones on Coomassie or amido-black stained gels (Granelli-Piperno and Reich, 1978). Analysis of the conditioned media from TCDD and B(a)P treated cultures, showed the presence of a single 54 kDa lytic band after staining. Evaluation of the relative sizes of the lytic bands generated by the conditioned media from treated cultures was performed by densitometric scanning. The analysis indicated that neither 10-100 nM TCDD, nor 10 μM B(a)P significantly altered fibrinolytic activity in conditioned media from cultures of RL95-2 cells compared to 0.1% (v/v) DMSO controls (Figure 4-7B).

Effects of TCDD and B(a)P on TIMP mRNA Expression in RL95-2 Cells

We next evaluated the steady state mRNA levels for the TIMPs because of their role in the regulation of proteases important in the degradation of matrix membrane proteins. Northern blot analysis was performed after 48 hr exposure of RL95-2 cultures to 10 nM TCDD and 10 μM B(a)P. Both TCDD and B(a)P treatments significantly increased the steady state levels of the 1 kb mRNA transcript for TIMP-1 relative to DMSO treated controls ($p < 0.05$); however, B(a)P was able to induce relatively higher steady state levels of TIMP-1 than was TCDD (Figure 4-8A & B). In contrast, a time course analysis of TIMP-2 mRNA levels after treatment of cultures with 10 nM TCDD did not demonstrate any significant change in the levels of either the 1.2 or 3.5 kb mRNA transcripts (Figure 4-9). Thus there appears to be a selective increase in the expression of steady state levels of TIMP-1 mRNA as opposed to TIMP-2 by TCDD and B(a)P exposure in the RL95-2 cell line.

Discussion

Rier et al (1993) demonstrated that the incidence and severity of endometriotic lesions in female rhesus monkeys was associated with their dietary exposure to TCDD in a dose-related manner. Study of a surgically-induced model of endometriosis in rodents similarly showed that the administration of TCDD and the pesticide methoxychlor (MTX) significantly promoted the growth of endometriotic sites (Cummings and Metcalfe, 1995; Cummings et al., 1996). In this regard, the physiological and molecular mechanisms whereby endometriotic tissue develops and persists outside of the uterine cavity and musculature are not well understood. On the basis of observed alterations in cellular and humoral immune function in endometriosis patients, it has been hypothesized that endometriosis may be the result of a decreased immune surveillance, recognition and destruction of misplaced endometrial tissue (Dmowski et al., 1994). Results of our

investigation into the potential alteration in immunological components by TCDD will be examined in Chapter five. The present chapter examined the data which relates to the potential role of TCDD and B(a)P in the alteration of factors involved in endometrial cell invasion and their potential contribution to the promotion of endometriotic lesions.

The data from the present study as presented in Figure 4-3 demonstrate that B(a)P produced a significant decrease in the cell invasive activity of the RL95-2 endometrial cell line. In contrast, TCDD exposure did not significantly alter the ability of the RL95-2 cells to invade matrigel membranes. Our data with B(a)P is consistent with epidemiological findings that cigarette smoking is correlated with significantly reduced incidence of the disease (Cramer et al., 1986; Mattorras et al., 1995). The fact that TCDD did not significantly alter the cell invasive activity of the exposed cultures implies that this agent may not act to promote endometriosis by enhancing the implantation of uterine cells, but may perhaps act at a later phase of growth and proliferation. Alternatively, the fact that the RL95-2 cell line is derived from a carcinoma may mean that the cell line already exhibits a maximal invasive phenotype. Consequently, it may be difficult to observe increases in the invasive ability of these cells after exposure to TCDD.

Several factors should be considered in the analysis of the ability of B(a)P to decrease overall cellular invasiveness as determined by this assay. These factors include the requirement that the endometrial cells first attach to the matrigel membrane prior to invasion. The possibility exists that B(a)P acts at the level of cellular attachment through a change in the expression of cell adhesion molecules. In this regard, we evaluated the ability of RL95-2 cells treated with TCDD and B(a)P to adhere to matrigel membranes. The degree of attachment produced by B(a)P-treated cultures was comparable to the degree of overall invasion, being significantly lower than that of TCDD or control cultures. The B(a)P levels used to treat the RL95-2 cultures are on the high end of that which would present in a heavy smoker. Furthermore, only viable cells, as determined by tryphan blue

exclusion were utilized in the invasion and attachment assays, therefore the results observed in these experiments are most likely not the result of B(a)P cytotoxicity.

These results are consistent with the observation that primary cells from endometriotic biopsies, but not normal endometrium, have been reported to exhibit the loss of E-cadherin expression (Gaetje et al., 1997), while a lack of β3 integrin expression was found to be closely correlated with a diagnosis for endometriosis (Lessey et al., 1994). In this regard, E-cadherin is regarded as an invasion suppressor, cell adhesion molecule. Secondly, the previous chapter presents evidence that B(a)P produced a state of growth arrest in these endometrial cell cultures under serum-free conditions, and markedly slowed their overall rate of proliferation in complete media. The invasion and attachment assays were performed after treatment of RL95-2 cultures in complete media. A reduced rate of proliferation produced by B(a)P could affect the ability of the cells to invade basement membrane if growth and proliferation are required for the elaboration of factors involved in the attachment/invasion process.

Based on the simplified schematic of the process of cellular invasion (Figure 4-1), we next evaluated the potential of TCDD and B(a)P to alter the expression of specific proteases and their inhibitors which could account mechanistically for the contributions of these environmental agents to the etiology of endometriosis. Urokinase plasminogen activator (uPA) is a serine protease which catalyses the conversion of plasminogen to plasmin, another protease which is itself able to play a role in an array of processes such as tissue growth and remodeling, tumor invasion and metastasis (Mayer, 1990). The ability of uPA to generate plasmin allows for the activation of members of the matrix metalloprotease family of enzymes capable of degrading extracellular matrix proteins like collagen and fibronectin (Stetler-Stevenson et al., 1993).

Our data provide evidence that TCDD, but not B(a)P, is able to significantly increase the expression of steady state mRNA levels of uPA in a time- and dose-dependent manner. Furthermore, this induction of uPA steady state mRNA levels appeared to be the

consequence of post-transcriptional processes as shown by the ability of CHX to produce a superinduction of the mRNA message. In this regard, TCDD may be altering the expression of protein(s) which are involved in the regulation of steady state uPA mRNA levels. For example, the decreased expression of a protein involved in degradation of uPA mRNA would result in an increased half-life for the uPA message, and could account for the observed effect of TCDD on uPA mRNA levels.

We next chose to evaluate whether this observable increase in mRNA was translated into enhanced fibrinolytic activity present in the conditioned media from endometrial cultures exposed to TCDD and B(a)P. Our experiments did not demonstrate any significant increase in the fibrinolytic activity of conditioned medium from either TCDD or B(a)P treated cultures. These observations led to the conclusion that if any differences existed, they may be present in the endometriotic tissues themselves. The fact that no increases in fibrinolytic activity were observed for TCDD treated cultures although there was a significant increase in steady state uPA mRNA expression could be the result of compartmentalization. Studies of cells in culture have shown that uPA can be compartmentalized, i.e bound to cell-surface receptors at focal contact points and remains active when bound to its membrane localized receptor. Hence increases in uPA activity in conditioned media might not be readily observed.

These data raise a number of interesting points of discussion. First, the ability of TCDD to increase the steady state level of uPA mRNA has previously been demonstrated in human keratinocytes, which further analysis showed to be the result of post-transcriptional regulation via an increase in the half life of the uPA message (Gaido and Maness, 1995). Immunohistochemical analysis of the levels of PA in the endometrium of women with endometriosis showed a variation in the levels in normal endometrium throughout the menstrual cycle, whereas endometriotic tissue maintained a consistently high level of immunoreactive PA (Fernández-Shaw et al., 1995). These results indicate a potentially more invasive nature of the endometriotic implants.

Alternatively, Malick (1982) developed the hypothesis that changes in fibrinolytic activity could contribute to the development of endometriosis. Malick suggested that decreased peritoneal fibrinolytic activity could be responsible for the adhesions seen in the disease because of a decreased capacity to lyse fibrin deposits which develop secondary to peritoneal injury. Human endometrial cells have been shown to release two major forms of PA, tissue-type (tPA) and urokinase (uPA), whose expression and release are regulated by progesterone, estrogen and EGF (Miyauchi et al., 1995a, b). The fibrin gel zymography assay works equally well for the evaluation of either tPA or uPA fibrinolytic activity (Granelli-Piperno and Reich, 1978). In our study, the lytic activity generated by the conditioned medium of RL95-2 endometrial cultures is centered around a 54 kDa band which corresponds to the molecular weight of uPA, rather than tPA which is a 70 kDa protein. The fact that our data show no increase in the actual fibrinolytic activity of the conditioned medium is supported by other workers who have failed to find any significant differences in the fibrinolytic activity of peritoneal fluid from women with endometriosis and/or pelvic adhesive disease compared to control patients (Batzofin et al., 1985; Dunselman et al., 1988).

The present study also examined the steady state level of mRNA expression of two members of the family of tissue inhibitors of matrix metalloproteases (TIMPs) after treatment of endometrial cultures with TCDD and B(a)P. TIMP-1 mRNA levels are increased by both TCDD and B(a)P exposure, with a greater level of induction by B(a)P compared to TCDD. In comparison, neither TCDD nor B(a)P significantly altered the levels of the two TIMP-2 mRNA transcripts. TIMPs belong to a family of proteins which inhibit collagenases and gelatinases, and an imbalance between proteinases and their activators and inhibitors has been implicated in a number of pathological states including tumor invasion, fibrosis and arthritis (Stetler-Stevenson et al., 1993). Manipulation of the balance between MMPs and TIMPs can induce or suppress cellular invasion, as demonstrated by the ability of the overexpression of TIMP-2 in human melanoma A2058

cells to modulate not only proteolysis of the extracellular matrix, but also the adhesive and spreading properties of the cells (Ray and Stetler-Stevenson, 1995).

The lack of change in the mRNA levels for TIMP-2 in our RL95-2 cells may not be at all surprising based on the recent characterization of the TIMP-2. This analysis has shown that TIMP-2 has several features observed in housekeeping genes, with mRNAs transcripts having longer half lives than that of β-actin (Hammani et al., 1996). This is in contrast to TIMP-1 and TIMP-3 which exhibit highly inducible levels of mRNA expression. The expression of TIMP-2 can be characterized as largely constitutive, in contrast to TIMP-1 and TIMP-3 both of which are highly inducible at the transcriptional level in response to phorbol esters and serum growth factors (Edwards et al., 1996). Thus TIMP-2 may play a major role in providing a stable basal level of inhibitory activity in tissues (Hammani et al., 1996). The ability of both TCDD and B(a)P to induce TIMP-1 mRNA levels would lend support to a reduced invasiveness for these cells, but such a phenomenon is observed only for B(a)P treated cultures. Cellular invasion is a multistep process, involving the net co-ordinated interaction of a number of genes and gene products. The increased TIMP-1 mRNA levels observed after TCDD and B(a)P treatment in this cell line may, therefore, not be readily interpretable on their own, as indicators of an alteration in overall cellular invasiveness.

In summary, B(a)P, but not TCDD, was shown to inhibit the ability of RL95-2 endometrial cultures to attach to and traverse matrigel membranes. TCDD, but not B(a)P, significantly increased the steady state levels of uPA mRNA, yet neither TCDD nor B(a)P altered the fibrinolytic activity of the conditioned medium from treated cultures. Finally, both TCDD and B(a)P enhanced the level of expression of TIMP-1 mRNA, but had no effect on TIMP-2 expression. Further work needs to be carried out in order to better characterize the overall effect of these environmental agents on cellular invasion and tissue remodeling factors and so elucidate their full potential role in uterine pathologies.
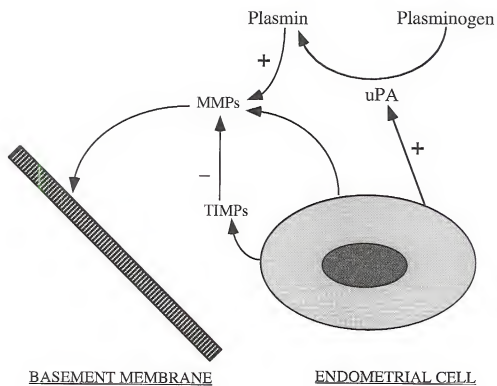
Figure 4-1. Illustration outlining the potential mechanism of invasion and implantation of endometrial cells.
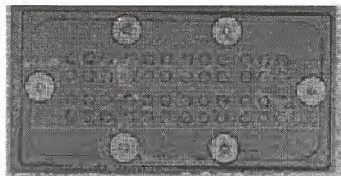
(A)

(B)

Figure 4-2. Diagram of a modified Boyden Chamber apparatus.
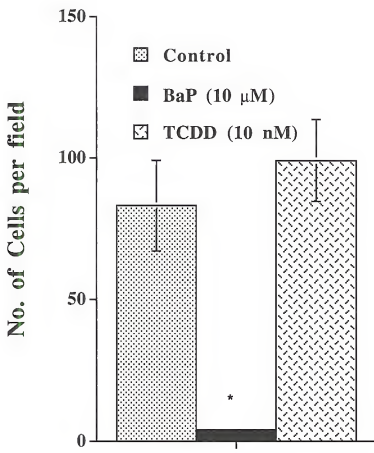(A) Cross-sectional view and (B) View fron above.

Figure 4-3. Quantitation of the effect of 10 nM TCDD and 10 μM B(a)P pre-treatment on the ability of RL95-2 cells to invade Matrigel membranes. Approximately 2 x 10⁴ cells were aliquoted into the bottom wells of the apparatus and allowed to attach to a 8 micron Matrigel-coated membrane for 90 min after inversion. Upper wells were loaded with complete media and cells allowed to migrate for 36 hr. Cells which migrated through the membrane were stained with Leukostat and counted under a light microscope. Data are expressed as the mean ± SEM of the number of migrating cells from three separate experiments. * P < 0.001 as compared to 0.1% DMSO controls.

(A).

(B).
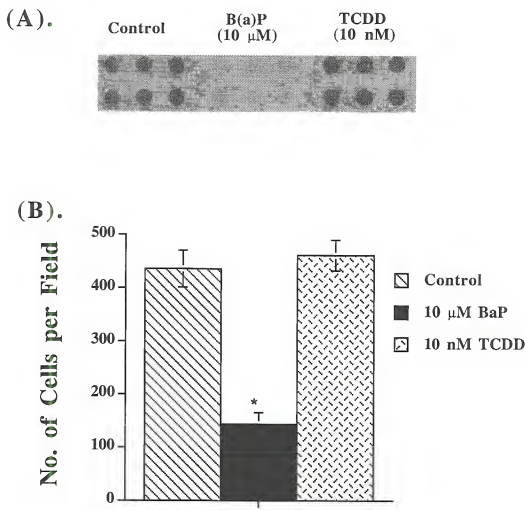

Figure 4-4. Quantitation of the effect of 10 nM TCDD and 10 μM B(a)P pre-treatment on the ability of RL95-2 cells to attach to Matrigel membranes. Approximately 5×10^3 cells were aliquoted into the bottom wells of the apparatus and allowed to attach to a 8 micron Matrigel-coated membrane for 2 hr after inversion. Cells which attached to the membrane were stained with Leukostat and counted under a light microscope. (A) Scanned image of treated RL95-2 cell attached to matrigel membranes. (B) Data expressed as the mean ± SEM of the number of attached cells from two separate experiments. * P < 0.001 as compared to 0.1% DMSO controls.

(A)

	6h		12h		24h		48h	
TCDD (10 nM)	-	+	-	+	-	+	-	+
uPA								

β-Actin

(B)

B(a)P (10 μM)   0   1   10

uPA

β-Actin
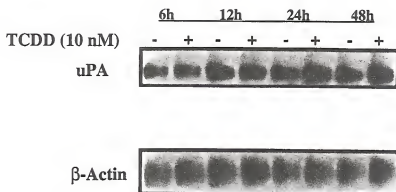
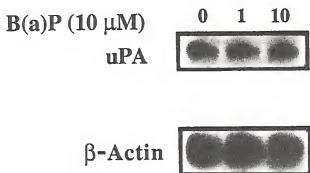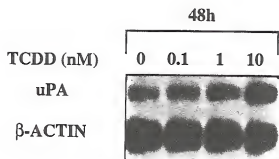Figure 4-5. Northern blot analysis of uPA mRNA. Cells were exposed to TCDD or B(a)P and total RNA isolated, denatured, blotted and hybridized with ^{32}P-labeled cDNA probes as described in Materials and Methods. (A) Representative blot of the time-dependence of the induction of uPA with 10 nM TCDD treatment. (B) Blot of the effect of B(a)P exposure on uPA mRNA levels in RL95-2 cells for 48 hr.

(A).

(B).

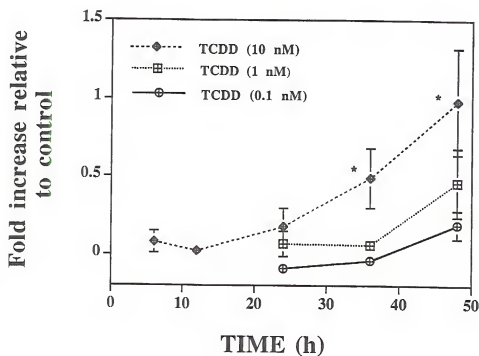Figure 4-6. Dose-dependent effect of TCDD on the expression of uPA mRNA in RL95-2 cells. (A) Northern blot analysis of total RNA after a 48 hr exposure to varying concentrations of TCDD. Analysis performed as described in Materials and Methods. (B) Graphical representation of the dose-dependent effect of TCDD on uPA mRNA levels. Data represent the mean scan intensity $\pm$ SEM of at least three experiments. * P < 0.05.
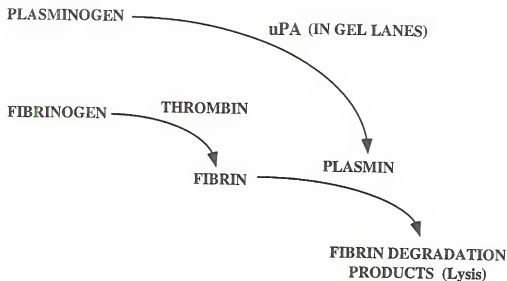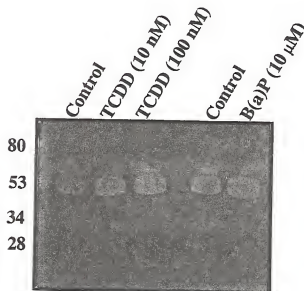
Figure 4-7. Evaluation of the fibrinolytic activity of the conditioned media from B(a)P and TCDD treated RL95-2 cultures. (A) An outline of the methodology involved in the fibrinolytic analytical technique. (B) Representative photograph of the lytic bands produced by the plasminogen activity of the conditioned media from the treated cultures.

(A).
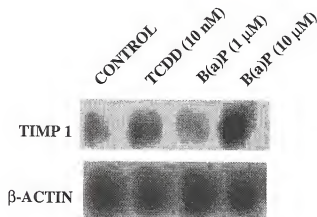
(B).
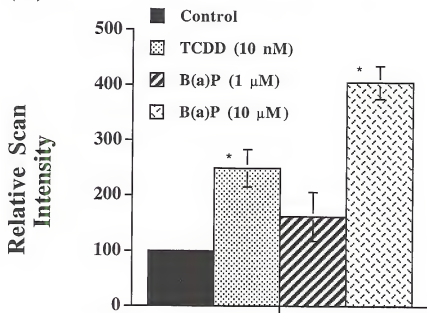
Figure 4-8. Northern analysis of TIMP-1 mRNA. RL95-2 cultures were exposed to TCDD, B(a)P or 0.1% DMSO for 48 hr after which total RNA was isolated, denatured, blotted and hybridized to ^{32}P-labeled cDNA probes as described under Materials and Methods. (A) Representative Northern blot probed with ^{32}P-TIMP-1. (B) Graphical representation of the scan intensity from treated cultures. Bars represent the mean ± SEM of at least three experiments. * P < 0.05

(A)
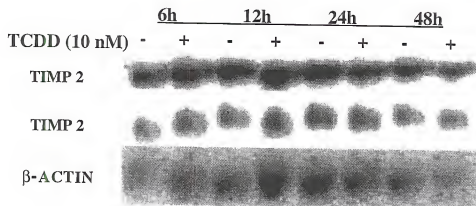
(B)
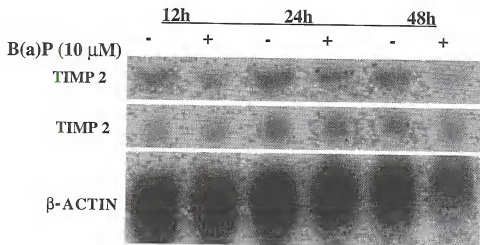
Figure 4-9. Northern analysis of TIMP-2 mRNA. RL92-2 cultures were exposed to (A) 10 nM TCDD, (B) 10 μM B(a)P, or 0.1% DMSO for 48 hr and total RNA isolated, denatured, blotted and hybridized to ^{32}P-labeled cDNA probes as described under Materials and Methods. (A) and (B) are representative Northern blot probed with ^{32}P-TIMP-2 generated from cultures treated as indicated. Blots show the presence of both the 1.2 and 3.5 kb mRNA transcripts for TIMP-2.

EFFECTS OF TCDD ON IL-1β AND TNFα IN A HUMAN ENDOMETRIAL CELL
LINE

Introduction

Normal endometrium undergoes predictable biochemical and histological changes in response to hormones throughout the menstrual cycle. Cytokines including IL-1 and TNFα are produced by cell populations within the uterine environment and may participate in growth and differentiation of the endometrium (Frank et al., 1995; Laird et al., 1996; Roby and Hunt, 1994; Simón et al., 1993). IL-1 and TNFα are among the pleiotropic growth factors which may act as local mediators of cellular communication in the uterine cavity (Hunt et al., 1992), with the IL-1 system having recently been demonstrated to play a role in the process of embryonic implantation (Simón et al., 1994). Dysregulation of the expression of these cytokines could potentially result in pathological disorders of the endometrium and uterine cavity and musculature.

The Sampson hypothesis postulates that endometriosis is a consequence of the implantation and growth of desquamated endometrial cells and fragments at extrauterine sites, yet this model does not take into account the prevalence of retrograde menstruation within the female population (Halme et al., 1984). An alternative theory is based on evidence that alterations in the normal immune system regulation may facilitate the implantation of endometrial fragments, thereby contributing to growth, and disease progression (Dmowski et al., 1994; Gleicher and Pratt, 1993; Rier et al., 1995). Evidence in support of this immunologic hypothesis comes from data showing evidence of elevated levels of inflammatory cell products such as IL-1, IL-5, IL-6, IL-8, IL-10, TNF-α and prostaglandins in peritoneal fluid as well as macrophage and macrophage-conditioned

media from endometriosis patients (Keenan et al., 1995; Koyama et al., 1993; Mori et al., 1991; Rana et al., 1996; Taketani et al., 1992). In this regard, danazol, a mainstay of the medical management of endometriosis, is able to suppress the production of IL-1 and TNF by human monocytes (Mori et al., 1990). Further evidence for a role for immunological factors in the maintenance of endometriotic implants is based on the ability of macrophages to secrete cytokines capable of influencing endometrial growth, as well as the observation that peritoneal fluid from women with endometriosis increases endometrial cell proliferation (Ramey and Archer, 1993). Alterations in cell-mediated and humoral immunity have also been noted in the disease (Dmowski et al., 1994; Gleicher, 1994).

TCDD is a well characterized immunotoxicant (Holsapple et al., 1996; Kerkvliet, 1995; Masten and Shiverick, 1995). TCDD has previously been shown to induce the expression of both IL-1β and TNFα *in vivo* (Fan et al., 1997) and *in vitro* in human keratinocytes (Sutter et al., 1991) and in MCF-7 breast cancer cells (Vogel and Abel, 1995). The ability of these compounds to alter cytokine expression could facilitate a role in the pathogenesis of uterine disease. The objective of this study was to evaluate the effects of TCDD on the expression of IL-1β and TNFα in a human endometrial cell culture model. The study examined the dose and time dependence of TCDD exposure with changes in cytokine mRNA levels.

Results

Effects of TCDD on IL-1β and TNF-α mRNA levels in RL95-2 Cells

TCDD exposure significantly increased the steady state level of mRNA for the IL-1β transcript relative to controls as evaluated by Northern blot analysis (Figure 5-1). Data show that 10 nM TCDD exposure of RL95-2 cultures resulted in a time-dependent induction of the expression of IL-1β mRNA which is observed as early as 6 hr after treatment. Maximal induction of about 5-fold occurred by 36 hr after TCDD treatment,

with levels remaining elevated above controls for the duration of the 48 hr assay period (Figure 5-2). Not only was the induction of IL-1β mRNA time-dependent, but it was also observed to be dose-dependent (Figure 5-1B). A representative Northern blot performed after a 24 hr treatment with concentrations of TCDD from 0.1-10 nM TCDD indicates that IL-1β mRNA is induced by TCDD exposures as low as 0.1 nM and is maximal by 10 nM TCDD. In comparison, RL95-2 exposure to TCDD led to a significant ($p < 0.05$), time-dependent induction of TNF-α mRNA steady state levels by 36 hr (Figure 5-3). Levels plateaued at this 36 hr time point and remained elevated relative to controls for the remainder of the 48 hr assay period (Figure 5-3B). Hence TCDD treatment resulted in significantly increased levels of mRNA for both IL-1β and TNF-α. However, the time course and relative levels of induction varied between both messages. IL-1β mRNA was significantly increased at an earlier time point as well as showing higher levels of induction at maximal levels relative to TNF-α (Figure 5-2 and 5-3). It warrants note that the late induction of TNF-α showed a time-dependency similar to that exhibited for uPA induction (Figure 4-5 and 5-3).

Effect of TCDD on the Rate of Transcription of CYP1A1, CYP1B1, uPA, and IL-1β mRNA in RL95-2 Cells

Nuclear runoff analyses were performed in order to determine whether the observed changes in uPA and IL-1β mRNA levels were the result of increased transcriptional activity. Nuclei were isolated from RL95-2 cells treated with 0.1% DMSO and 10 nM TCDD and analyzed for the levels of nascent mRNA transcripts. Data in Figure 5-4 demonstrate that CYP1A1 transcription is significantly increased 5-fold by TCDD, whereas no effect was observed on CYP1B1, uPA or IL-1β transcripts.

Furthermore, we also utilized the protein synthesis inhibitor cycloheximide (CHX) to investigate whether the induction of uPA, TNF-α and IL-1β mRNA was dependent upon

the synthesis of other proteins. As shown in Figure 5-5, the exposure of cultures of RL95-2 cells to CHX in the presence or absence of TCDD led to the superinduction of uPA, TNF-α and IL-1β mRNA levels, implying that the levels of these mRNA are tightly regulated and dependent upon *de novo* protein synthesis. Gaido et al (1995) has recently shown that TCDD does in fact regulate the level of uPA mRNA in a human keratinocyte cell line via a post transcriptional mechanism. Thus evidence indicates that TCDD is not acting to increase the steady state levels of mRNAs for IL-1β, TNF-α and uPA by directly altering the rate of mRNA transcription.

<u>Discussion</u>

The present study investigated the potential of TCDD to alter the expression of cytokine/growth factor genes which may play a role of the etiology of endometriosis. Our data indicate that TCDD is able to induce the expression of mRNA for both IL-1β and TNF-α in the RL95-2 cell line. In the case of IL-1β, this induction is both time- and dose-dependent. For TNF-α, a time-dependent increase in steady state levels of mRNA was observed with TCDD exposure. This induction of IL-1β and TNF-α by TCDD has been demonstrated previously in human keratinocytes (Sutter et al., 1991), a breast cancer cell line (Vogel and Abdel, 1995) and, more recently, in rat hepatic tissue after in vivo exposure (Fang et al., 1997). Significantly, this is the first observation of these responses in uterine cells and may lead to a better understanding of the potential actions of TCDD and related compounds in uterine disease etiology.

IL-1β and TNF-α are pleiotropic cytokines secreted by a variety of cell types. Their role in mediating responses in the female reproductive system and during the course of gestation has recently come under greater scrutiny (Simón et al., 1994; Tabibzadeh, 1991). Many of the processes occurring during menstruation are reminiscent of the inflammatory response in terms of cellular proliferation, ischemic necrosis, stromal granulocyte infiltration and angiogenesis (Tabibzadeh, 1991). Both cytokines have been detected in

human and rodent uterine tissue, with their expression apparently being cyclically regulated by estrogen and progesterone levels (Frank et al., 1995; Hunt et al., 1992; Laird et al., 1996; Roby and Hunt, 1994). The pleiotropic nature of these polypeptides, has generated difficulty in defining their precise roles in the uterine environment.

The immunological hypothesis of endometrial promotion, has resulted in study of IL-1β and TNF for their potential contribution to the pathology of the disease. Increased numbers of activated macrophages have been detected in the peritoneal and tubal fluids of infertile women with endometriosis (Haney et al., 1983). Activated macrophages secrete IL-1β, and increased levels of this cytokine have been found in the peritoneal fluid of women with endometriosis above that from healthy women or those treated for the disease (Keenan et al., 1995; Mori et al., 1991; Rana et al., 1996). Danazol, one of the mainstays of the medical management of endometriosis, results in decreased levels of these cytokines in the peritoneal fluid of treated subjects (Mori et al., 1990; Taketani et al., 1992).

The outcome of an inflammatory cascade could lead to the synthesis of new connective tissue and adhesion formation as well as increased vascularity as the result of angiogenesis, processes which could be mediated by activated macrophages through their elaboration of pro-inflammatory cytokines. Induction of angiogenesis, procoagulant activity and mitogenic action on fibroblasts are among the actions of IL-1β and TNF (Tabibzadeh, 1991). IL-1 has been shown to stimulate collagen deposition and fibrinogen formation (Posthlewaite et al., 1984), which might account for the incidence of fibrosis and adhesions observed in advanced stages of endometriosis. Peritoneal fluid from endometriotic women has been shown to have a toxic effect on mouse embryo development (Taketani et al., 1992), an observation which has been reproduced with IL-1β (Fakih et al., 1987), although some controversy is involved in the reproducibility of this data (Schneider et al., 1989).

The data we have presented demonstrates a time-dependence of the induction of messages for both IL-1β and TNF, with IL-1β induction being observed by 6 hr and TNF-

α being apparent by 36 hr. This observation is interesting in light of the fact that IL-1β has been shown to stimulate the production of TNF-α in endometrial cells and cytotrophoblasts in culture (Knöfler et al., 1997; Laird et al., 1996). It has been suggested that cytokines are integrated into an intricate network that operates by coordinated regulation of their expression. Our observation of the time-dependent induction of mRNA levels for IL-1β and TNF may be a manifestation of this phenomenon.

The fact that IL-1β and TNF are characterized primarily as immune system modulators, supports their potential role in the pathogenesis of uterine disorders like endometriosis and may relate to effects on cellular attachment and invasion processes. IL-1β has recently been shown to be involved in the ability of murine embryos to be successfully implanted (Simon et al., 1994), while the adherence of endometrial stromal cells to mesothelial cells was significantly increased by pretreatment of mesothelial cells with TNF-α (Zhang et al., 1993b). The latter observation may be associated with the ability of TNF to alter the expression of molecules involved in adhesion interactions such as cadherin and β-catenin (Tabibzadeh et al., 1995). The role of these pleiotropic cytokines in the process of implantation is further supported by their ability to alter the expression of matrix metalloproteases (MMPs), enzymes involved in the degradation of basement membrane molecules to allow for tissue remodeling (Librach et al., 1994; Sato et al., 1996).

The present study also examined the possibility that the induction of mRNA for these cytokines could potentially be regulated at the transcriptional level. To this end, we utilized the method of nuclear runoff analysis to examine the translation of nascent transcripts from TCDD-treated cultures of RL95-2 cells. Our data indicate that while CYP1A1 steady state mRNA levels were indeed increased as a result of increased transcription rates, IL-1β and uPA mRNA levels were not (Figure 5-4). Both IL-1 and TNF have been reported to be regulated at both the transcriptional and post-transcriptional level (Fenton, 1992; Tabibzadeh, 1991). Data in Figure 5-5 which show the

superinduction of IL-1β mRNA levels after exposure of RL95-2 cultures to CHX, a protein synthesis inhibitor, lends support to a post-transcriptional mechanism of regulation. TNF-α, for example, is thought to be primarily controlled at the post-transcriptional level, and cells expressing the TNF-α message may not necessarily translate it into product (Sariban et al., 1988). In fact, TNF-α release from cytotrophoblast cultures was demonstrated to be independent of the induction of its mRNA levels (Knöfler et al., 1997).

In summary, TCDD induced the expression of the steady state levels of mRNA for IL-1β and TNF-α in a time-dependent manner. Analysis of the transcription of IL-1β demonstrated that the induction was not a result of increased transcription rates, but likely the consequence of post-transcriptional phenomena. Thus, chronic inflammatory changes in endometriosis may be mediated at least in part by IL-1β and TNF-α. Our data showing increased expression of IL-1β and TNF-α mRNA after TCDD exposure opens the possibility TCDD may also play a role not only in the invasive potential of desquamated endometrium, but also in the infertility associated with the disease.

(A)
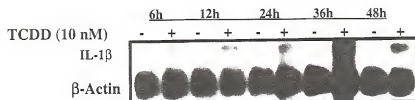
(B)
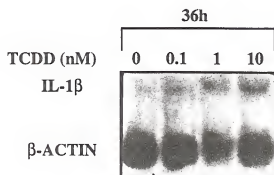
Figure 5-1. Northern blot analysis of IL-1β showing the dose- and time-dependence of its induction with TCDD. (A) Representative Northern blot showing the time-dependence of the induction of IL-1β mRNA with 10 nM TCDD exposure. (B) A representative Northern blot showing the dose-dependence of IL-1β mRNA induction after a 36 hr exposure to varying concentrations of TCDD. Forty µg of total RNA was denatured, blotted and hybridized with ^{32}P-labeled cDNA as described under Materials and Methods.
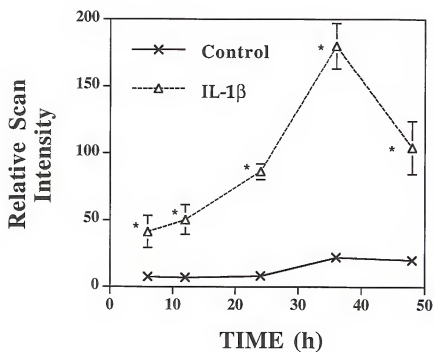
Figure 5-2. Graphical analysis of the induction of IL-1β mRNA in RL95-2 cultures treated with 10 nM TCDD (Δ), relative to 0.1% (v/v) DMSO (x) controls. Data represent the mean ± SEM from five experiments. * P< 0.001 compared to controls.

(A)
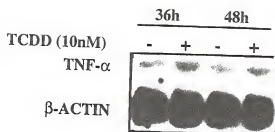
(B)
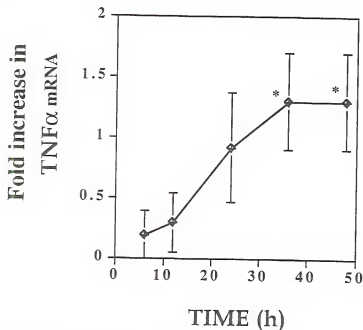
Figure 5-3. The effect of TCDD exposure on TNF-α mRNA expression in RL95-2 cells. (A) Northern analysis of TNF-α showing a time-dependent induction with TCDD exposure. Total RNA, 40 µg, was denatured, blotted and hybridized with ^{32}P labeled cDNA for TNF-α as described in Materials and Methods. (B) Graphical analysis of the induction of TNF-α mRNA with time of exposure to TCDD. Values represent the mean ± SEM of three experiments. * P < 0.05.
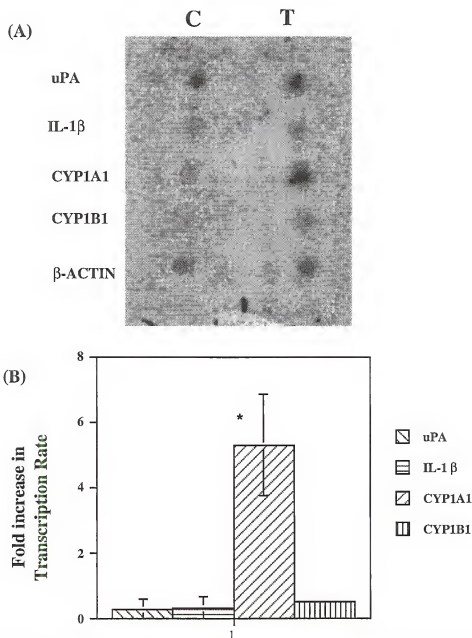
Figure 5-4. Effect of TCDD on the rate of IL-1β and uPA transcription. Actively proliferating RL95-2 cultures were treated with either 10 nM TCDD (T) or 0.1% (v/v) DMSO (C). Nuclei were isolated after 44 hr and nuclear runoffs performed as described under methods. β-actin was used as a loading control and CYP1A1 as a positive control for transcriptional induction by TCDD. (A) Representative autoradiogram of a runoff analysis for transcriptional induction. (B) Results of the desitometric analysis of the band intensities after normalization to β-actin indicating relative changes in rates of transcription. Each bar represents the mean ± SEM of at least three experiments for 10 nM TCDD treatment. * P< 0.01 compared to β-actin.
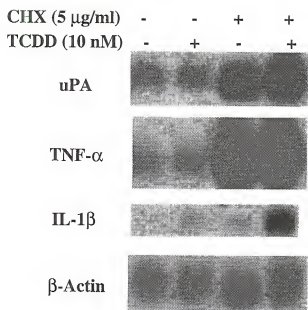
Figure 5-5. Northern analysis showing superinduction of mRNA for uPA, TNF-α and IL-1β by cycloheximide in RL95-2 cells. Fifty μg of total RNA from RL95-2 cultures was isolated after 12 hr treatment with the protein synthesis inhibitor cycloheximide (CHX), in the presence or absence of 10 nM TCDD. Bands were visualized by autoradiography after membranes were probed with ³²P-labelled cDNA probes.

CHAPTER 6
EXPRESSION AND PURIFICATION OF RECOMBINANT AND NATIVE
PROLACTIN-LIKE PROTEINS B AND C

Introduction

The rodent placenta is a rich source of proteins which are structurally related to pituitary PRL. To date, fifteen of these PRL-like proteins have been identified and shown to exhibit a temporal and cell-specific pattern of expression. It has been suggested that these proteins are members of a superfamily of hormones and cytokines, the helix-bundle-peptide (HBP) hormones, with shared structural features and mechanisms of action. The HBP structure consists of four long α-helices arranged in antiparallel fashion (Horseman and Yu-Lee 1994) with family members including GH, PRL, erythropoietin and the interleukins. Based on their homology to PRL, some of these placental proteins have been characterized and shown to possess PRL-like bioactivity (Cohick et al., 1995; Colosi et al., 1987a, b; Deb et al., 1991a; Robertson et al., 1982, 1994). The biological activity of a number of these proteins, however, remains as yet undetermined.

Our laboratory had previously identified four major rat placental proteins secreted into conditioned media from placental explant cultures. These proteins were identified by N-terminal sequence analysis to be related to the PRL/GH family (Ogilvie et al., 1990a). A cDNA coding for a protein virtually identical to that of Proteins 2 and 4 was later isolated by Deb et al (1991b) and designated PLP-C based on an already established nomenclature (Duckworth et al., 1986b, 1988). Our laboratory has studied several models of low birth weight in pregnant rats and found that maternal xenobiotic exposure and protein malnutrition, respectively, are associated with decreased placental growth, vascular development and intrauterine growth retardation (IUGR). Moreover, growth-retarded

78

placentas showed decreased expression of protein and mRNA for PLP-B (Conliffe et al., 1995; Shiverick et al., 1991). Thus, previous evidence supports an association between impaired placental growth and decreased expression of PLP-B and PLP-C proteins.

In an effort to identify the biological role of PLP-B and PLP-C in placental growth, our laboratory initiated efforts to purify native forms of the placental protein, as well as attempt recombinant expression of PLP-B and PLP-C proteins in both bacterial and mammalian systems. This section of the study details the expression and purification of recombinant PLP-C using two different bacterial constructs, as well as the mammalian expression and purification of PLP-B. Recombinant PLP-C was further used to generate a polyclonal antisera which was essential for immunoaffinity purification of the native protein from conditioned media of basal zone explant cultures. Finally, this section also details the use of an antipeptide antisera previously generated against the C-terminus of PLP-B (Ogilvie et al., 1990b) for purification of native protein.

Results

Purification of Recombinant hCAII-PLP-C

In the hCA-II fusion vector p0304 (Van Heeke et al., 1993), the DNA sequence encoding the recombinant protein is linked to hCA-II through a cleavage recognition sequence for enterokinase. In order to achieve the expression and subsequent release of PLP-C from the hCAII fusion protein, it was necessary to subclone the DNA fragment encoding PLP-C into an *Eco*RV site located adjacent to the enterokinase cleavage site such that a *Dra*I site would be present immediately adjacent to the codon specifying the first amino acid in the mature form of PLP-C (Conliffe et al., 1994). Aliquots from different stages in the purification of recombinant PLP-C were separated on 10% SDS-PA gels under reducing conditions and proteins were visualized by Coomassie staining (Figure 6-1). The predicted molecular weight of the hCAII-PLP-C fusion protein is 53,620 (hCAII,

MW 31,234; PLP-C, MW 22,386). As shown in Figure 6-1, lanes A and D, a band corresponding to a protein of 53 kDa was purified from the bacterial extract. The linkage of PLP-C to hCAII allowed for rapid purification of the fusion construct utilizing a pAMBS resin with high affinity for hCAII. The 53 kDa band comprised approximately 15% of the Coomassie blue-stained affinity-purified protein (Figure 6-1, lane D).

Western immunoblot analysis using a PLP-C antipeptide antisera revealed a broad band of 31-36 kDa and another band of 53 kDa in the cell lysate and pAMBS-purified fusion protein preparations (Figure 6-2, left panel, lanes A and D.). The immunoreactive protein of 53 kDa corresponded to the calculated molecular weight of the fusion construct of hCAII-PLP-C and also cross reacted with hCAII antisera (Figure 6-2, right panel, lane D). The broad 31-36 kDa immunoreactive band comprised about 75% of the purified fusion protein and the molecular weight was confirmed by Mass Spectrometry to be 31-36 kDa. These lower molecular weight bands were initially thought to be hCAII-PLP-C proteolytic products resulting from the action of bacterial proteases released during cell lysis. The addition of the protease inhibitors, phenylmethylsulfonyl fluoride, leupeptin, aprotinin and EDTA during cell lysis, however, did not reduce the staining intensity of these bands (data not shown). These bands also stained positively with a hCAII antisera (Figure 6-2, right panel, lanes A and D) and had an N-terminal sequence, His-His-Trp-Gly-Tyr-Gly-Lys-His-Asn-Gly, which was identical to that of hCAII. Thus, data indicate that the immunoreactive bands of 53 kDa, as well as the 31-36 kDa were hCAII fusion proteins.

Enterokinase cleavage of the affinity-purified fusion protein produced an immunoreactive protein band of approximately 25 kDa (Figure 6-3, lanes A and B), corresponding to the calculated molecular weight of PLP-C. As judged by Coomassie blue staining, the 25 kDa protein was highly enriched by affinity purification (Fig.6-3, lane B). Recombinant PLP-C yield varied with different preparations. Enterokinase cleavage of PLP-C from its fusion partner produced approximately 15% recombinant PLP-C. Efforts

were made to maximize cleavage conditions, by increasing enzyme concentration and incubation times, as well as by changing Ca^{2+} concentration, but there was not a significant increase in yield of PLP-C protein.

Recombinant Expression and Purification of pET22b(+)His-PLP-C

Because of the poor yield of the original hCAII-PLP-C construct in generating recombinant PLP-C, we undertook the construction of an alternative bacterial expression system (Figure 6-4). Expression of recombinant PLP-C with a polyhistidine tag at its N-terminus was chosen because this system allowed for purification using the ability of poly-histidine to bind divalent metal ions even under denaturing conditions (Hochuli et al., 1987). To achieve the expression and subsequent release of PLP-C protein from the pET22b(+) expression system, it was necessary to anneal a polyhistidine primer to the 5' end of the PLP-C cDNA from the original hCAII-PLP-C vector which included the intact enterokinase linker region. The resulting construct was then ligated into the *NdeI/NotI* sites of the pET22b(+) expression vector as described under Materials and Methods and as outlined in Figure 6-4. The presence and orientation of the desired insert was determined by restriction enzyme digestion with *NdeI* which would result in the release of an approximate 700 bp fragment of the PLP-C cDNA as shown in Figure 6-5.

Aliquots from different steps in the purification of recombinant PLP-C from the pET22b(+)His-PLP-C system were separated by 10% SDS-PAGE under reducing conditions and proteins visualized by Coomassie staining (Figure 6-6). The recombinant protein of approximately 27 kDa is seen in lanes A, D, E, G and H of Figure 6-6A. The figure shows steps from two different purification schemes. Lanes A-D are from the soluble fraction of the bacterial pellet. The recovery of mostly insoluble protein (lanes E-H) required detergent solubilization of the pellet in N-laurylsarcosine (detergent), followed by refolding under alkaline conditions at room temperature overnight (Luck et al., 1991,

1992). The His-PLP-C protein was then isolated from the supernatant by affinity purification on immobilized nickel cation columns. Figure 6-6B is a Coomassie stained gel which illustrates the results of the enterokinase cleavage of the His-PLP-C protein to generate the full length recombinant protein. Lane A represents the full length protein after enterokinase cleavage of the His-PLP-C product (Lane B). The 25 kDa protein was electroblotted onto PVDF membrane and amino terminal sequence analysis revealed a major sequence of Ile-Pro-Ala-Cys-Met-Val-Glu. This sequence was identical to that of amino acids 1-7 of PLP-C (Deb et al., 1991b, c; Ogilvie et al., 1990b). A minor species, less than 25%, was also detected, with N-terminal sequence corresponding to residues 6-12 of PLP-C.

Expression of Recombinant PLP-B

The expression of reconbinant PLP-B in a mammalian system was chosen because of the fact that the native protein exists only as a glycosylated species (Duckworth et al., 1986b). The complete cDNA coding sequence for PLP-B was blunt-end ligated into the pMXSND expression vector as outlined in Figure 6-7. This vector has previously proven useful in the generation of recombinant proteins for other members of the PRL/GH family (Cohick et al., 1997; Deb et al., 1993; Lee and Nathans, 1988). Blunt-ended ligation of the PLP-B cDNA was necessary because there was only a single *Xho*I cloning site in the pMXSND vector with no other compatible site for 'sticky ended' ligation with the PLP-B cDNA.

*Bam*HI diagnostic restriction endonuclease digests were performed to determine the presence of the PLP-B insert in the correct orientation. Figure 6-8 illustrates the results of digests performed upon putative plasmids containing the PLP-B insert. Lanes 8 and 10 show results of the diagnostic digests of plasmids incorporating the pMXSND-PLP-B insert in the incorrect and correct orientation, respectively. CHOK1 cells were transfected with the pMXSND-PLP-B plasmid and stable transfectants selected by culture in the

presence of G418. Stable clones were selected after expansion by probing for the presence of mRNA species which hybridized to ^{32}P-PLP-B cDNA on Northern blot analysis (Figure 6-9). Clones 4, 5 and 7 were observed to give positive hybridization signals and were consequently expanded by growth in large cultures with G418 and MTX selection. PLP-B secretion was induced by the culture of stable transfectants in the presence of $CdCl_2$ which makes use of the metallothionein promoter in the pMXSND vector. Western immunoblot analysis of concentrated conditioned media from these stably transfected cultures was performed using an antipeptide antisera against the C-terminus of PLP-B (Ogilvie et al., 1990b) and demonstrated the presence of immunoreactive protein of approximately 30 kDa (Figure 6-10) which corresponded in size to native PLP-B protein (Ogilvie et al., 1990a).

Purification and Western Immunoblot Analysis of Native PLP-B and PLP-C

Native PLP-B and PLP-C were purified from the conditioned media of gestation day 18 rat placental (basal zone) explant cultures. The utility of antisera generated against these proteins (Ogilvie et al., 1900b; Conliffe et al., 1994) in immunoaffinity chromatography was demonstrated. The purified native preparations of PLP-B and PLP-C were characterized by two dimensional SDS-PAGE. As seen in Figure 6-11, PLP-B is present as a series of 30 kDa subforms with pI ranging between 6.1-6.6, as observed by silver staining (Figure 6-11A) and Western immunoblot analysis (Figure 6-11B). The anti-PLP-C column retained species of 25 kDa and 29 kDa with pI ranging from 5.8-6.2, as observed on silver stained two dimensional SDS-PAGE (Figure 6-12A) and confirmed by Western immunoblot analysis (Figure 6-12B). These data are in agreement with the previously reported size and pI characteristics of secreted PLP-B (Ogilvie et al., 1990a) and PLP-C (Conliffe et al., 1994).

Discussion

The present study describes the recombinant expression and purification of PLP-B and PLP-C, two members of the PRL-GH family produced by the rat placenta. Furthermore, the study also describes the purification of these native proteins from placental explant cultures by immunoaffinity chromatography with the use of antibodies generated against PLP-B and PLP-C. Recombinant PLP-C was initially expressed as a fusion partner with human carbonic anhydrase (hCAII) in JM109(DE3) *E. coli* bacteria. The usefulness of hCAII has been previously demonstrated as a partner in fusion protein expression constructs with *E.coli* F1-ATPase ε subunit and asparagine synthetase A, respectively, (Hinchman and Schuster, 1992; Van Heeke et al., 1993). In the present study, linkage of PLP-C to hCAII allowed the fusion protein to be purified to homogeneity in a single step on a sulfonamide affinity resin. The desired 53.4 kDa fusion protein was produced in our expression system in addition to several other hCAII fusion proteins of 31-36 kDa. These lower molecular weight proteins may represent premature translation termination products of PLP-C. In this regard, it is possible that chain termination might be due to differences in codon usage between mammalian and bacterial systems.

In the expression system for the hCAII fusion protein, the recombinant PLP-C protein is released from its fusion partner by enterokinase cleavage. Although enterokinase digestion of hCAII-PLP-C released recombinant PLP-C, the yield was found to be highly variable. Because the enterokinase products of the 31-36 kDa bands would not exceed 6 kDa, it is expected that they are excluded from the preparation during the centriprep concentration step which has a 10 kDa cutoff membrane. In this regard, Hinchman and Schuster (1992) were unable to obtain any cleaved product in the expression of asparagine synthetase A. In contrast, release of PLP-C from the His-PLP-C construct was achieved with a high level of consistency. This disparity in release of the recombinant protein may be attributed to differences in enterokinase preparations or, alternatively, to folding of the recombinant protein which might interfere with cleavage.

The difficulties encountered in the use of the hCAII fusion system for the expression of recombinant PLP-C necessitated the development of an alternative expression construct. The enterokinase PLP-C sequence was excised from the original hCAII-PLP-C construct and ligated to a polyhistidine tract at the N-terminal region of PLP-C upstream of the enterokinase region. The presence of the polyhistidine region allowed for purification based on histidine affinity for divalent metal ions by passage over a nickel column. The removal of the approximately 3 kDa N-terminal polyhistidine extension was effected by incubation with enterokinase which released the recombinant PLP-C. This polyhistidine fragment was assumed to be excluded after dialysis with a 6 kDa membrane.

The recombinant PLP-C protein isolated from the two bacterial systems had similar molecular weight and N-terminal amino acid sequence identity with that of native placental PLP-C (Deb et al., 1991b; Ogilvie et al., 1990b). A minor species corresponding to PLP-C beginning at residue 7 was also detected in the expression system. Studies describing the expression of recombinant rat placental PLP-A in a mammalian cell line also identified minor species representing N-terminally truncated isoforms of PLP-A (Deb et al., 1993). The antiserum generated against recombinant PLP-C demonstrated cross reactivity with the 25 kDa non-glycosylated and the 29 kDa glycosylated forms of PLP-C in Western immunoblot analysis.

This study also described the expression of PLP-B as a recombinant protein utilizing a mammalian expression vector pMXSND. This vector has previously been shown to be effective in the expression of other members of the PRL-GH family from both rat and murine sources (Cohick et al., 1997; Colosi et al., 1988; Deb et al., 1993; Lee and Nathans, 1988). The choice of mammalian expression was also important based on the fact that the native protein has been detected only as a 30 kDa glycosylated species (Ogilvie et al., 1990b; Duckworth et al., 1988). The lack of cloning sites within pMXSND necessitated that the PLP-B cDNA be blunt-end ligated into the vector and that transformed colonies be screened for the presence of the PLP-B cDNA insert in the correct orientation

by restriction enzyme digestion prior to transfection into CHOK1 cells. Stable transfectants were selected after Northern blot analysis using a ^{32}P-labeled cDNA probe. The conditioned medium of stably transfected clonal colonies exhibited the presence of a 30 kDa immunoreactive band in Western immunoblot analysis corresponding in molecular weight to native placental PLP-B protein.

This study further described the purification of native preparations of PLP-B and PLP-C from placental explant conditioned medium in immunoaffinity chromatography using antibodies generated against PLP-B and PLP-C. Purified native PLP-B preparations were separated by 2D-SDS polyacrylamide gel electrophoresis and Western immunoblot analysis demonstrated the presence of 30 kDa molecular weight species with similar molecular weight and pI to that of the native protein (Ogilvie et al., 1990b). Similarly, purified native PLP-C preparations showed the presence of several immunoreactive species in Western immunoblot analysis as well as on silver stained gels. The presence of both the 25 kDa non-glycosylated and 29 kDa glycosylated forms of native placental PLP-C has been previously characterized (Deb et al., 1991b). The other minor species in these preparations may represent products of genes which are closely related to PLP-B and PLP-C or, alternatively, are post-translational isoforms of these proteins.

In summary, PLP-B and PLP-C were both successfully expressed as recombinant proteins in mammalian and bacterial systems, respectively. Placental PLP-C is expressed as both non-glycosylated and glycosylated species. Our efforts to express PLP-C in mammalian systems were not successful. In this regard, other researchers in the field have similarly demonstrated an inability to recombinantly express PLP-C in a mammalian system (M. Soares, personal communication). Antiserum was generated against PLP-C recombinant protein and, along with an antipeptide antiserum against PLP-B, utilized in the immunoaffinity purification of the native proteins from placental explant conditioned medium. The availability of these protein preparations should allow for their further characterization.
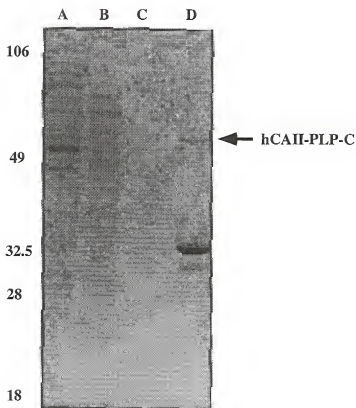
Figure 6-1. SDS-PAGE analysis of the purification of hCAII-PLP-C fusion protein. Aliquots from different steps in the purification of hCAII-PLP-C fusion protein were separated on 10% polyacrylamide gels under reducing conditions and visualized by Coomassie brilliant blue staining. Lane A, 50 μg cell lysate prior to affinity purification; lane B, 50 μg cell lysate after affinity purification; lane C, 50 μg pAMBS column wash; lane D, 15 μg affinity purified fusion protein; molecular weight standards (x 10^3) kDa are indicated on the left.
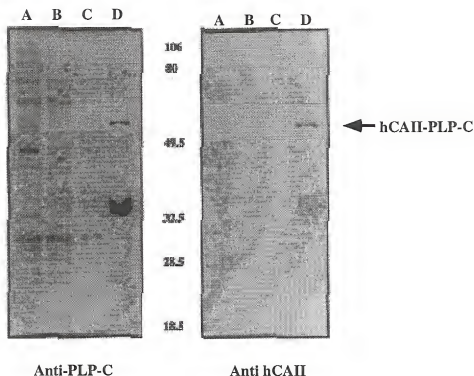
Figure 6-2. Western immunoblot analysis of hCAII-PLP-C fusion protein preparations following each purification step. Samples were separated on 10% polyacrylamide gels under reducing conditions and analysed by immunoblotting using antisera (1:800) raised against PLP-C oligopeptide (Fig.6-2, left panel) or hCAII (Fig. 6-2, right panel). Lane A, 50 μg cell lysate prior to affinity purification; lane B, 50 μg cell lysate after affinity purification; lane C, 50 μg pAMBS column wash; lane D, 15 μg affinity purified fusion protein; molecular weight standards (x 10³) kDa are indicated on the left.
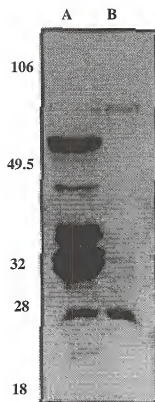
Figure 6-3. SDS-PAGE analysis of hCAII-PLP-C fusion protein cleaved by enterokinase. The recombinant PLP-C was separated from hCAII by pAMBS affinity chromatography, and then concentrated and dialysed. Samples were separated on 10% gels under reducing conditions and stained with Coomassie brilliant blue. Lane A, 25 μg enterokinase-cleaved proteins; lane B, 3.5 μg purified recombinant PLP-C; molecular weight standards (x 10^3) kDa are indicated on the left.
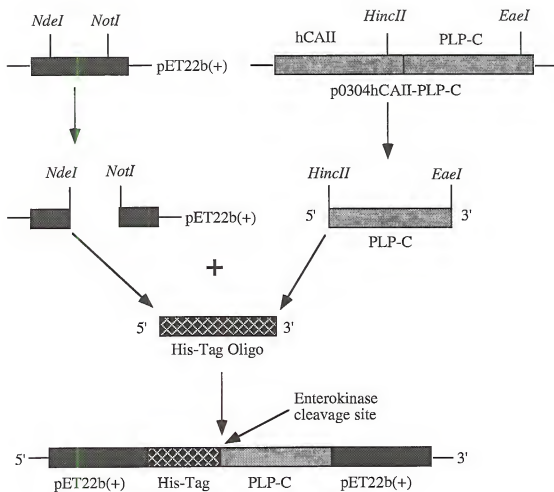
Figure 6-4. Schematic of the construction of a vector expressing PLP-C with an N-terminal polyhistidine tract. The top line shows the pET22b(+) plasmid and the hCAII-PLP-C vector being cut with restriction enzymes as indicated. The middle lines show the three way ligation of pET22b(+), an oligo encoding a 9 mer polyhistidine tract and the PLP-C cDNA fragment excised from the hCAII-PLP-C vector. The bottom line shows the structure of the pET22b(+)His-PLP-C vector and the position of the enterokinase cleavage site at the junction of the histidine tract and the PLP-C cDNA insert.

Figure 6-5. NdeI diagnostic digests of plasmids from JM109 bacterial colonies transformed with the pET22b(+)His-PLP-C ligation mix. Photograph shows the diagnostic cleavage of five plasmids with NdeI for the presence of the PLP-C insert. (-) indicates an uncut plasmid, while (+) indicates the plasmid after overnight incubation with restriction enzyme NdeI. Plasmid number 2 in the fifth lane from the left shows the presence of the expected 700 bp PLP-C insert after NdeI digestion.

Figure 6-6. SDS-PAGE analysis of the steps in the purification of pET22b(+)His-PLP-C protein. Aliquots were separated on 10% polyacrylamide gels under reducing conditions and visualized by Coomassie brilliant blue staining. (A). Lane A, 10 µg cell lysate prior to passage over the nickel affinity column; lane B, 10 µg cell lysate after affinity purification; lane C, 10 µg column wash; lane D, 3 µg affinity purified protein; Lane E, 10 µg of the bacterial pellet solubilized in N-lauroylsarcosine (detergent), lane F, 10 µg solubilized after passage over the nickel column; lane G, 3 µg column eluate; lane D, 3 µg affinity purified protein after dialysis. (B). SDS-PAGE of enterokinase cleaved pET22b(+)His-PLP-C protein. The protein was digested with enterokinase (Lane A) and recombinant PLP-C separated from the His tag by dialysis (Lane B). Molecular weight standards (x10^3) are indicated on the left.

(6A)

(6B)
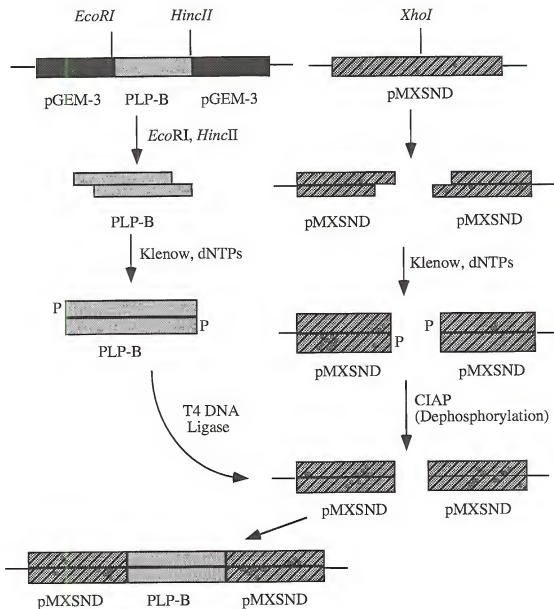
Figure 6-7. Schematic of the construction of a mammalian vector expressing PLP-B. The top line shows the pMXSND vector and the pGEM3-PLP-B plasmid being cut with the relevant restriction enzymes as indicated. The second and third lines show the creation of blunt-ended pMXSND and the PLP-B cDNA fragment preparatory to ligation after dephosphorylation of the pMXSND vector. The bottom line shows the structure of the pMXSND-PLP-B vector.
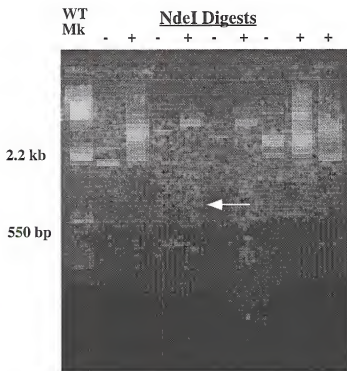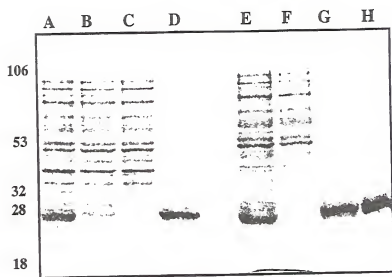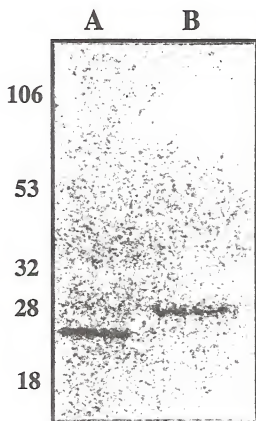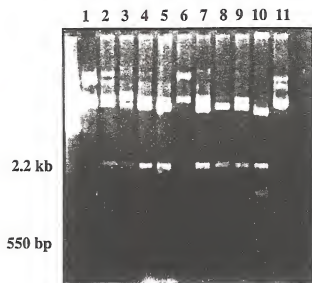
Figure 6-8. BamHI diagnostic digests of plasmids from JM109 bacterial colonies transformed with the pMXSND-PLP-B ligation mix. Photograph shows the diagnostic cleavage of eleven plasmids with BamHI for the presence of the PLP-B insert in the correct orintation. The plasmid in lane number 7 and 10 from the left shows the presence of the PLP-B insert after BamHI digestion. Lane 10 possesses the insert in the correct orientation as seen by the expected 1250 bp fragment as opposed to the 750 bp fragment in lane 7.
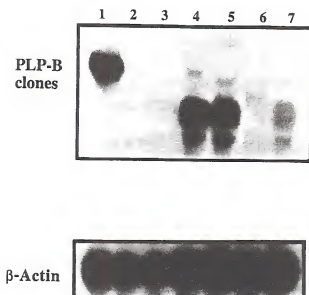
Figure 6-9. Northern blot analysis of total RNA from clonal colonies of CHOKI cells transfected with the pMXSND-PLP-B expression vector. RNA was denatured, electrophoresed, transferred onto nylon membranes and hybridized with ^{32}P-PLP-B. Colonies 4, 5 and 7 were amplified and used for the purification of PLP-B from conditioned medium.
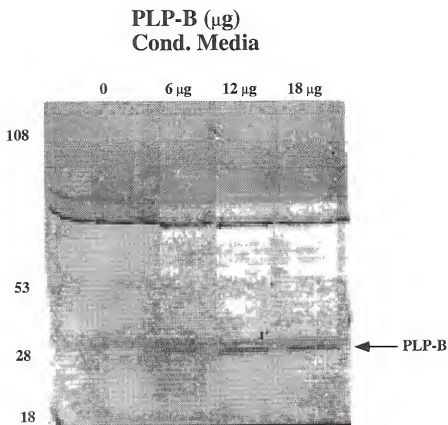
Figure 6-10. Western immunoblot analyses of PLP-B levels in the conditioned media from stably transfected CHOK1 cells. Samples were separated on 10% polyacrylamide gels under reducing conditions and analysed by immunoblotting using antisera (1:1000) raised against PLP-B oligopeptide Increasing quantities of conditioned media from CHOKI cells stably transfected with the pMXSND-PLP-B construct. Arrow indicates the location of the PLP-B immunoreactive band. 0, represents conditioned medium which had passed through a 10 kDa cutoff membrane used in the protein concentrating step. Molecular weight standards (x 10^3) are indicated on the left.

A

7 ——————— pH ——————— 4

45K

29K

20K

Native PLP-B

B

7 ——————— pH ——————— 4

45 K

29 K

20 K
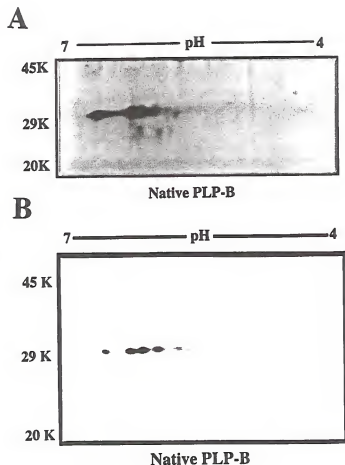
Native PLP-B

Figure 6-11. Two dimensional SDS-PAGE analyses of PLP-B preparations. Native protein was immunopurified by immunoaffinity chromatography and electrophoresed on 2D gels and analysed by silver staining for PLP-B (Figure 6-11A). Samples were run on duplicate gels and electroblotted onto nitrocellulose and analysed by Western immunostaining with antisera to PLP-B (Figure 6-11B). Blots were probed with an antipeptide antiserum against PLP-B at 1:1000 dilution, followed by incubation with horse-radish peroxidase conjugated with anti-rabbit IgG. Bands were visualized by incubation with 3-amino-9-ethylcarbazole in the presence of 0.015% hydrogen peroxide.

A

7 ———————— pH ———————— 4

45K

29K

20K

Native PLP-C

B

7 ———————— pH ———————— 4

45K

29K

20K
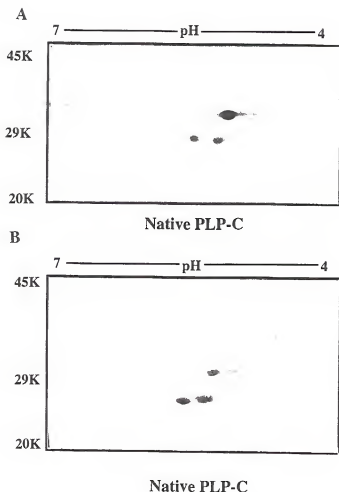
Native PLP-C

Figure 6-12. Two dimensional SDS-PAGE analysis of PLP-C preparations. Native protein was purified by immunoaffinity chromatography and electrophoresed on 2D gels and analysed by silver staining for PLP-C (Figure 6-12A). Samples were run on duplicate gels and electroblotted onto nitrocellulose and analysed by Western immunostaining with antisera to PLP-C (Figure 6-12B). Blots were probed with an antiserum against recombinant PLP-C at 1:1000 dilution, followed by incubation with horse-radish peroxidase conjugated with anti-rabbit IgG. Bands were visualized by incubation with 3-amino-9-ethylcarbazole in the presence of 0.015% hydrogen peroxide.

CHAPTER 7
CHARACTERIZATION OF NATIVE AND RECOMBINANT PROLACTIN-LIKE PROTEINS B AND C

Introduction

The recombinant proteins PLP-B and PLP-C were expressed and purified as described in the previous chapter. In addition, native forms of the PLP-B and PLP-C proteins were purified from the conditioned medium of basal zone explant cultures by immunoaffinity chromatography. This chapter discusses the evaluation of the biological activity of the purified proteins. Both the potential lactogenic and angiogenic activities have been evaluated using standard in vitro biological assay systems.

Firstly, PRL is known to exhibit a broad range of distinct physiological actions important in the course of reproduction (Handwerger et al., 1992). The secretion by the placenta of high levels of placental lactogens and PRL-related proteins during pregnancy has led to the hypothesis that these proteins are of importance in the placental-fetal growth axis. Secondly, during the course of gestation, fetal growth and development require enhanced placental transport and uterine blood flow which is dependent on placental neovascularization (Reynolds et al., 1986). Two members of the placental PRL-GH family, proliferin (PLF) and proliferin related protein (PRP), have recently been shown to display angiogenic and angiolytic activity, respectively (Jackson et al., 1994). Our laboratory has previously studied several animal models for low birth weight and found that impaired placental growth and vascular development was correlated with decreased secretion of PLP-B and PLP-C proteins by rat placental tissue (Conliffe et al., 1995; Shiverick et al., 1991). These observations led us to further investigate the potential for these two proteins, PLP-B and PLP-C to exhibit lactogenic and angiogenic activities.

Results

Effects of Native PLP-B and PLP-C and Recombinant PLP-C on Nb2 Lymphoma Cell Proliferation

Native PLP-B and PLP-C were evaluated for lactogenic activity utilizing a classic cell proliferation bioassay technique (Gout et al., 1980; Tanaka et al., 1980) with Nb2 lymphoma cells. Ovine PRL was used as a positive control and produced a dose-dependent stimulation of ^{3}H-thymidine uptake with an EC_{50} of approximately 0.1 nM. Both native PLP-B and PLP-C exhibited some lactogenic activity but only at concentrations above 100 ng/ml (Figure 7-1). The maximal activity of native PLP-B and PLP-C observed was, therefore, at concentrations which were at least 3 orders of magnitude greater than that of standard oPRL controls. The two PLP-B and PLP-C preparations were further analyzed for the presence of PRL and PL-II by Western immunoblot analysis in order to determine whether residual amounts of these known lactogenic placental proteins in the native PLP preparations could account for the observed activity. In data not shown, immunoreactive bands corresponding to the PL-II or PRL proteins were not observed, nor were these bands observed on silver-stained 2D gels of these PLP proteins. By comparison to the native preparation, recombinant PLP-C purified from bacterial expression systems did not exhibit any lactogenic activity relative to the ovine PRL control in this bioassay (Figure 7-2). Thus, evidence suggests that the lactogenic activity expressed by the native PLP-C protein preparation may be a consequence of glycosylation which was absent from the recombinant protein expressed in bacteria.

Effects of Placental Conditioned Medium, and Native PLP-B and PLP-C on Endothelial Cell Migration

Gestation day 18 placental tissue was cultured in serum-free medium for 24 hr and the conditioned medium evaluated for the ability to stimulate the directional migration of

human retinal endothelial cells as a measure of angiogenic activity. Medium from the cultures of placental (basal zone) tissue markedly stimulated the number of endothelial cells/well migrating through the collagen-coated membranes. The stimulating activity in placental conditioned media was highly significant relative to that exhibited by the 10% FBS positive controls (Figure 7-3), being 5-7 fold greater than that of the negative controls ($p < 0.001$). In addition, the stimulatory activity of placental conditioned media appears to be dose-related in that the response was greater with exposure to 250 µg compared to 50 µg of secreted protein.

In the cell migration assay, neither native PLP-B nor PLP-C preparations exhibited any significant angiogenic activity, even at concentrations up to 5 µg/ml (Figure 7-4). These data are representative of three experiments with different preparations of native PLP-B and PLP-C. The results indicate that day 18 basal zone conditioned medium possesses secreted, soluble angiogenic factor(s), whereas native PLP-B and PLP-C immunopurified from this media do not exhibit any angiogenic activity as measured by chemotactic activity towards endothelial cells.

<u>Discussion</u>

The secretion by the placenta of high levels of placental lactogens and PRL-related proteins during the course of pregnancy has led to the hypothesis that they may be of importance in the placental-fetal growth axis. Hence there is great interest in determining whether or not these newly discovered proteins were "PRL-like" in their ability to bind the PRL receptor or to exhibit similar bioactivities. Studies characterizing these rodent placental proteins have demonstrated that some of them do in fact exhibit PRL-like activities in their ability to produce lactogenic responses in rat Nb2 lymphoma cells (Gout et al., 1980) and in mammary gland epithelial cell differentiation (Handwerger et al., 1984;, Soares et al., 1983; Southard et al., 1986). Mouse and rat PL-I, II and Iv all exhibit such

activity, and rPL-I has been reported to be even more stimulatory than ovine PRL in the Nb2 assay.

As members of the PRL-GH family, both native PLP-B and PLP-C as well as recombinant PLP-C were evaluated for potential lactogenic bioactivity. The native protein preparations exhibited some detectable activity relative to that of ovine PRL in the Nb2 lymphoma bioassay, but at concentrations which were several orders of magnitude greater than that for the EC_{50} of ovine PRL. In this regard, the native preparations of PLP-B and PLP-C were further evaluated for the presence of contaminating residual amounts of PRL and PL-II (known lactogenic proteins) by Western immunoblot analysis and silver staining (data not shown) of 2D gels. The results, however, were negative for the presence of either known lactogenic protein. The presence of measurable lactogenic activity in native PLP-C led us to express a recombinant form of the protein for further evaluation of biological activity. When analyzed in the Nb2 lymphoma bioassay, however, recombinant PLP-C had no mitogenic activity. Furthermore, in data not shown, neither native PLP-B nor PLP-C were able to compete effectively for PRL binding to the long form of the PRL-receptor, nor were recombinant forms of the two proteins able to compete for binding to the rat PRL receptor (K. Shiverick, personal communication).

An analysis of the potential PLP-B and PLP-C three dimensional structure would lend support to these findings. The structure of the PRL-GH family of proteins, based on the X-ray crystallographic analysis of GH and mutagenesis studies in PRL, is thought to be composed of an up-up-down-down, four α-helical bundle motif, linked by interconnecting loops (Abdel-Meguid et al., 1987; de Vos et al., 1992; Goffin et al., 1992, 1993, 1994, 1996; Somers et al., 1994). As a consequence of the placental PRL-like proteins being folded to form this four α helix type structure, the amino acid residues exposed on their surfaces will vary since the helical regions are not highly conserved, with the exception of helix 4. This probably allows for variations in the properties available for interaction with other macromolecules (Goffin et al., 1996; Southard and Talamantes, 1991), this being true

for the lactogenic as well as non-lactogenic members of the family. Mutational analysis of the lactogenic mouse PL-II (mPL-II), along with sequence comparisons with the other members of the placental PRL family, led to the localization of several residues essential for maximal receptor binding and bioactivity (Davis and Linzer, 1989). These residues, Arg19, Arg171, Arg175, Lys185, Asn195 on mPRL, map to helix 1 and 4 of the putative four α helical folding pattern.

For the rat placental proteins of unknown biological activity, PLP-B lacks two of the key lactogenic residues while PLP-C has none of them. The fact that recombinant PLP-C did not exhibit the lactogenic activity observed for the native protein may also be a consequence of the lack of glycosylation of the recombinantly expressed bacterial protein. Alternatively, native preparations possessed some undetected soluble factor with significant lactogenic activity. Croze et al (1990) isolated a cDNA from rat decidua identical to the previously identified placental PLP-B cDNA. The expression of decidual PLP-B mRNA early in gestation after implantation to midgestation prompted Croze and co-workers (1990) to suggest that PLP-B could be a candidate gene which encodes the decidual luteotrophin of pregnancy. This protein has been shown to specifically interact with the luteal PRL receptor and stimulate the corpus luteum to produce progesterone (Jayatilak et al., 1989). The low potency of lactogenic response by native PLP-B and absence of high affinity binding to the PRL receptor (data not shown) is evidence that PLP-B is not the decidual luteotrophic protein.

Jackson et al (1994) has recently demonstrated that the midgestation mouse placenta secretes an angiogenic activity that corresponds primarily to PLF, followed by a factor that inhibits angiogenesis which is PRP. Recombinant PLF and PRP were further found to modulate the migration of endothelial cells in vitro and neovascularization in vivo (Jackson et al., 1994; Volpert et al., 1996). Previous analysis of the receptor binding activity of recombinant PLF showed a specific association with the insulin-like growth factor II/mannose 6-phosphate (M6P) receptor (Lee and Nathans, 1988). More recent data appear

to indicate that PLF manifests its angiogenic activity as a result of its interaction with this receptor (Volpert et al., 1996). Data from our laboratory indicated that both the mRNA and protein for placental PLP-B were significantly decreased in a low birth weight rat model where reduced placental vascular development was also observed (Conliffe et al., 1995). These observations led to the evaluation of native PLP-B and PLP-C for potential angiogenic activity in an attempt to elucidate their role in fetal-placental development.

Our data clearly show that gestation day 18 rat placenta secretes soluble factors which significantly stimulate endothelial cell migration. Our finding that late gestation rat placenta expresses predominantly stimulatory factors is in contrast with the report of Jackson et al (1994) that conditioned medium from late gestation mouse placental cultures was inhibitory to endothelial cell migration. The difference may be a reflection of our use of the basal zone (junctional zone) tissue for explant culture as opposed to the whole placenta which includes the labyrinth zone (Davies and Glasser, 1968). An additional possibility is that endothelial cells isolated from human retina differ from bovine adrenal capillaries in their responsiveness to chemotactic factors. In this regard, research on bovine models shows that the fetal placental tissue produces factors which inhibit endothelial cell migration and proliferation throughout the course of gestation (Reynolds and Redmer, 1995). Thus some evidence suggests that where these factors may function to limit vascular development is within the maternal placental vasculature.

The present study has also found that PLP-B and PLP-C immunopurified from the conditioned media did not show chemotactic activity towards endothelial cells. Thus it appears that other soluble angiogenic factors are released by the rat placental explants, which may include insulin-like growth factors (IGF) (Grant et al., 1987, 1992), as well as basic fibroblast growth factor (Hondermarck et al., 1990).

In summary, native PLP-B and PLP-C purified from rat placental conditioned media using immunoaffinity chromatography exhibit some lactogenic activity at concentrations above 100 ng/ml. In comparison, recombinant PLP-C purified from a

bacterial expression system shows no significant lactogenic activity in Nb2 lymphoma bioassays. Furthermore, neither native PLP-B nor PLP-C appear to be the rat homologues for PLF as factors which stimulate neovascularization of the placenta.
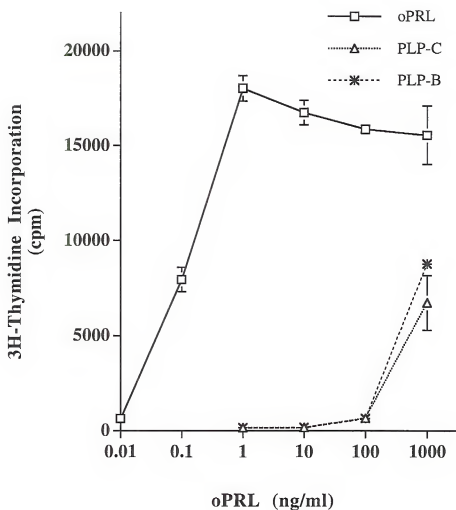
Figure 7-1. Effects of native preparations of PLP-B and PLP-C on Nb2 lymphoma cell proliferation. Cells (5 x 10^4), were treated in triplicate wells with varying concentrations of ovine PRL ($\square$), PLP-B (*) or PLP-C (Δ) at 37oC for 44 hr. Cells were harvested after a 4 hr incubation with ^{3}H-thymidine. Mean values ± SEM for two experiments are presented.
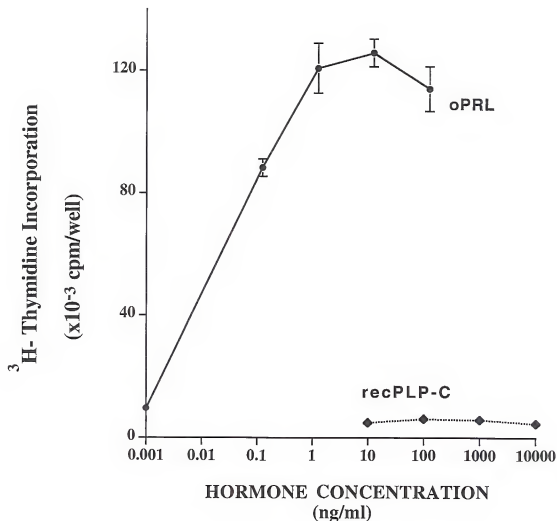
Figure 7-2. Effect of recombinant PLP-C on Nb2 lymphoma cell proliferation. Cells (8×10^4) were treated in triplicate with varying concentrations of ovine PRL (●) and recombinant PLP-C (♦) at 37°C for 44 hr. ^{3}H-thymidine incorporation was determined after a 4 hr labelling period. Mean values ± SEM for three experiments are presented.
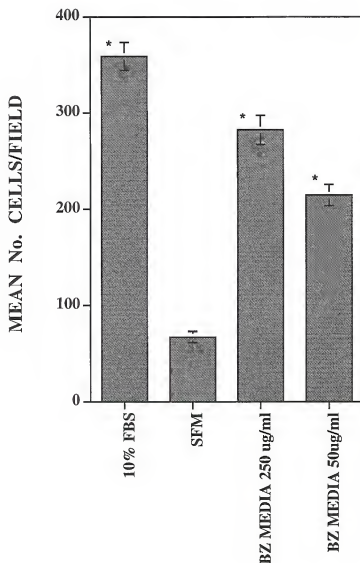
Figure 7-3. Quantitation of the angiogenic effect of day 18 placental (basal zone) conditioned medium on retinal endothelial cell migration. Details are as described in Materials and Methods. Upper wells were loaded with FBS, serum-free, or placental conditioned medium. Data are expressed as mean ± SEM of the number of migrating cells from three separate experiments. * P< 0.001 compared to serum-free media (SFM).
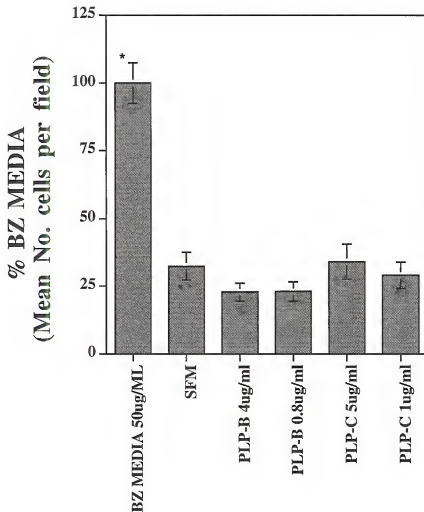
Figure 7-4. Quantitation of the angiogenic effect of gestation day 18 placental (Basal Zone) conditioned media, native PLP-B and native PLP-C on retinal endothelial cell migration. Details are as described in Materials and Methods. Upper wells were loaded with BZ medium and PLP-B or PLP-C proteins. Data are expressed as mean ± SEM of the number of migrating cells from three separate experiments. * P< 0.001 compared to serum-free media (SFM).

CHAPTER 8
CONCLUSIONS AND FUTURE DIRECTIONS

There has been growing public concern about the ability of environmental contaminants to contribute to adverse effects upon human health. This study grew out of the desire to investigate the potential mechanisms by which these agents may play a role in placental growth and development, and uterine disease pathologies, with particular regard to endometriosis. The objectives of this study were two-fold. Firstly, we investigated the cellular and molecular processes which could be altered by B(a)P and TCDD, two AhR agonists, in a human endometrial cell line, RL95-2. Secondly, this study attempted to characterize the potential role(s) of two placental proteins designated PLP-B and PLP-C whose expression has been shown to be altered in low birth weight animal models and by exposure to βNF. The conclusions which may be derived from this study, as well as the potential directions of future research efforts are described below.

The first part of this study demonstrated that both TCDD and B(a)P were able to induce the expression of members of the cytochrome P450 family, an action classically shown to be a consequence of AhR activation (Whitlock, 1993). B(a)P (a major contaminant in cigarette smoke), but not TCDD, is able to significantly decrease the expression of immunoreactive EGF receptor protein in RL95-2 cell cultures. A down-modulation of the EGF receptor has previously been observed after both B(a)P and TCDD exposure of human keratinocytes (Hudson et al., 1985). Our results indicate that receptor down-modulation is not directly correlated with CYP1A1 induction and it is possible that reactive metabolites have a role to play in the process, since B(a)P, in contrast to TCDD is readily metabolized to a series of reactive intermediates able to bind cellular macromolecules (Gelbion 1980; Leadon et al., 1988).

111

Furthermore, B(a)P, but not TCDD, was able to significantly decrease the proliferation of the endometrial adenocarcinoma cells. Endometriosis is an estrogen responsive disease, and estrogen actions on the uterus may be mediated through growth factors like EGF (Lingham et al., 1988; Nelson et al., 1991). Our data correlate with the etiological association of cigarette smoking with a decreased incidence of endometriosis. The role of reactive metabolites of B(a)P as a potential mechanism of EGF receptor down-modulation should be examined by analysis of RL95-2 cell cultures for metabolic products. Furthermore, the use of an antagonist to the AhR would also aid in determining whether or not the effects observed on the EGF receptor and/or the formation of B(a)P metabolites are mediated as a result of receptor activation.

This study next evaluated the potential role of genes and gene products which could contribute to the elaboration of changes in the invasive phenotype of these endometrial cells as a mechanism of disease promotion. TCDD, but not B(a)P, was observed to increase the steady state levels of uPA mRNA. Neither compound, however, was able to significantly alter the fibrinolytic activity of the conditioned medium of treated cells, as evaluated by fibrin zymography. Similarly, both compounds were able to increase the expression of TIMP-1 mRNA, but were without effect on TIMP-2 expression levels.

B(a)P, but not TCDD, was able to significantly decrease the overall ability of treated cells to invade matrigel membranes. The fact that cellular invasiveness was decreased by B(a)P correlates with epidemiological data regarding this disorder, however, the TIMP-1 data from our study does not. Our interpretation of the invasion data may need to take into consideration the experimental design of the assay in which the cells invade through an inverted membrane system. As a consequence of this, the observed inability of B(a)P treated cells to attach to the matrigel membrane would account for the observed B(a)P invasion data, an outcome which brings into consideration the role of adhesion factors in the invasion process. Thus an investigation of the B(a)P effects on potential alteration in the expression of members of the integrin and cadherin family may be

warranted, presently an area of research which may be gaining momentum in the field of endometriosis (Lessey et al., 1994; van der Linden et al., 1994). The fact that B(a)P resulted in a significant growth arrest of our endometrial cell cultures may also contribute to the observation of reduced invasive activity by these cells. Finally, the overall invasiveness of these cells may be the net result of alterations in the expression of other members of the MMP and TIMP families. An investigation of the potential changes in the expression of these genes and gene products would contribute to a better understanding of the control of the invasion process in uterine cells.

This part of the study also demonstrated that TCDD was able to significantly increase the expression of IL-1β and TNF-α, cytokines which potentially could contribute to the proinflammatory processes observed in endometriosis (Koyama et al., 1993; Ramey et al., 1993; Rana et al., 1996). The time course of TCDD induction of these responses may be important in that IL-1β has been shown to increase the secretion of TNFα from cytotrophoblast cell cultures, albeit without a change in mRNA levels for TNFα (Knöfler et al., 1997). These genes are regulated at a number of levels, especially at the post-transcriptional level in the case of TNF-α (Tabibzadeh, 1991). Thus it would be worthwhile to examine whether the observed changes in mRNA are manifested as increased levels of protein expression for these cytokines.

The fact that TNFα has been shown to alter the ability of cells from primary endometrial cultures to attach to basement mesothelial cells (Zhang et al., 1993b), as well as regulate the dyscohesion of epithelial cells (Tabibzadeh et al., 1995), may indicate a role for this cytokine in the modulation of adhesion factor expression. Furthermore, the observation of increased levels of prostaglandins in the peritoneal fluid of endometriotic patients may be the result of cytokine mediation. Prostaglandins have been implicated in the increase in endometrial vascular permeability which occurs during the implantation process (Chen et al., 1995). The potential of TCDD and B(a)P treated RL95-2 cultures to

release prostaglandins as a consequence of enhanced cytokine production can also be investigated.

The alterations produced by TCDD and B(a)P treatment of the RL95-2 endometrial carcinoma cells are summarized in Figure 8-1. As outlined, TCDD tends to stimulate what could be characterized as proinflammatory responses in these cells, while B(a)P produces decreased growth and decreased invasive potential. Further work, however, needs to be done to more completely characterize the overall responses of these cells to these prototypical environmental agents.

The second part of this study attempted to characterize the biological activities of two proteins secreted by the rat placenta during pregnancy. PLP-B and PLP-C were successfully purified from the conditioned medium of placental explant cultures using antibodies generated against the proteins for immunoaffinity chromatography. The native proteins exhibited molecular weights, pI and immunoreactivity similar to that of the previously characterized placental proteins (Deb et al., 1991b; Ogilvie et al., 1990b). Both PLP-B and PLP-C were also expressed as recombinant proteins in mammalian and bacterial expression systems, respectively. This is the first successful expression of recombinant PLP-C since attempts to express it in mammalian host systems have been unsuccessful.

Evaluation of the biological activity of the native PLP-B and PLP-C preparations demonstrated that both protein preparations exhibited low levels of lactogenic activity; however, this lactogenic activity was not observed for the recombinant PLP-C. The lack of activity by recombinant PLP-C may be a consequence of glycosylation, but this is presently difficult to determine due to the inability to express PLP-C in mammalian host systems. The placental conditioned medium, but not native PLP-B or PLP-C, exhibited significant angiogenic activity as evaluated by the ability of these preparations to stimulate the chemotaxis of human retinal endothelial cells. Future studies could utilize the purified recombinant proteins in order to localize their binding sites after [^{125}I] labeling.

Crosslinking studies could be performed with [^{125}I] labeled probe in an attempt to characterize the receptors for these proteins. The fact that PLP-B and PLP-C are major secretory placental proteins present during the second half of pregnancy argues for a physiological role during this period.

Epidemiology

CIGARETTE SMOKING

↓ Endometriosis

DIOXIN

↑ Endometriosis

Gene Expression

B(a)P

↓ Proliferation

↓ EGFR

↓ Invasion/Attachment

↑ TIMP-1

TCDD

↑ IL-1β

↑ TNF-α

↑ uPA

↑ TIMP-1

Phenotype

OVERALL

↓ Proliferation
Invasion/Attachment

OVERALL

↑ Proinflammatory
Responses

Figure 8-1. Comparison of the epidemiological factors in the incidence of endometriosis with alterations in gene expression in the endometrial cell line RL95-2.

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BIOGRAPHICAL SKETCH

Grantley Dexter Charles was born on December 15th 1967, in San Fernando, Trinidad. Despite being raised Baptist he spent his formative middle and high school under the tutelage of Irish Catholic monks (Presentation Brotherhood) at a parochial Catholic Grammar School. Upon graduation he worked as a substitute elementary school teacher for a year before entering the University of the West Indies in St. Augustine, Trinidad in 1987. There, after a three year travail, he received his B.Sc. degree in the Natural Sciences with a major in Chemistry. After a one semester stint as a middle and high school teacher, he came to the United States in January 1991, to pursue his then dream of a Master's Degree in Forensic Science at the John Jay College of Criminal Justice (CUNY) in New York. His interest being stirred by drug pharmacology and toxicology, he applied to and was accepted into the Department of Pharmacology and Therapeutics at the University of Florida in Fall 1992. There he gravitated to the laboratory of Dr. Kathleen Shiverick where for the course of his graduate study he has worked in the area of reproductive and developmental toxicology. He has accepted a postdoctoral position at The Dow Chemical Company in Midland, Michigan, in the laboratory of Dr. Edward Carney, Project Leader for the Developmental and Reproductive Toxicology (DART) Group.

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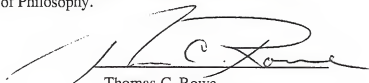
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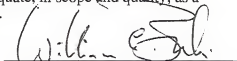
Thomas C. Rowe
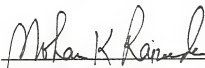Associate Professor of Pharmacology
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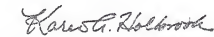
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